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ANNUAL REVIEW OF MEDICINE

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PREFACE

The editors have not attempted to produce here a summary of all of the new data and ideas which have developed in the past year throughout the broad field of internal medicine. Rather, as in the case of previous issues, this volume contains review articles which treat in depth various areas in which there have been remarkable recent advances in information and insight. A wide range of subject matter is covered, and the topics have been selected both for their general interest and for their intellectual and scientific importance. Some chapters, as the one on myasthenia gravis and the thymus, describe the culmination of many years of research by many investigators. Others, as the one on antigenic reversion in human cancer, describe work which is more recent in its origin and development.

The editors are grateful to the authors who have so generously contributed of their time and knowledge, to the George Banta Company, Inc. and the excellence of printing and skill which they bring to the production of these volumes, and to the Assistant Editor, Miss Beryl V. Daniel, who has presided with her usual effectiveness over the conception, gestation, and parturition of this volume.

THE EDITORIAL COMMITTEE

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PROINSULIN[1]

7000

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INTRODUCTION

Until 1967 it was widely accepted that insulin synthesis occurred within pancreatic β cells through combination of two separately synthesized polypeptide chains (1–3). In fact, in vitro experiments with separated natural or synthetic chains had shown relatively efficient resynthesis of insulin, especially in the presence of directing groups (4) or the enzyme, glutathione insulin transhydrogenase (5). Furthermore, the experiments of Humbel (3) provided indirect evidence for the separate parallel synthesis of the two chains of angler fish insulin on separate ribosomal units, followed by their combination. As an extension of this theory, Konikova, Morenkova & Kritzman (6) suggested that insulin was assembled from small oligopeptides, some of which contained cystine to provide the disulfide bridges. However, despite this evidence, the idea that the insulin molecule may be synthesized in vivo as a single chain precursor remained attractive. In 1965, Givol and co-workers (7) suggested that the amino acid sequence of the single chain proteins ribonuclease and chymotrypsinogen directed the folding of the molecule so as to permit the disulfide bonds to form spontaneously. After cleavage of their polypeptide chains at one or more sites, the stability of the proteins under conditions of disulfide-sulfhydryl exchange was markedly diminished in similar fashion to that which had been observed with insulin. By analogy, therefore, these authors suggested that the insulin chains might be formed as part of a larger single polypeptide from which insulin could be released by proteolysis after the native conformation had been established (7). However, Wang & Carpenter (8) had searched for such an insulin precursor in pancreatic extracts, without success and concluded that if present, it comprised less than 10 percent of the total insulin in the pancreas.

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BIOSYNTHETIC STUDIES

IDENTIFICATION OF PROINSULIN

Experiments leading to the discovery of proinsulin were begun in 1965 with an investigation of insulin biosynthesis in an islet cell adenoma which was removed from a patient with severe hypoglycemia (9). When slices from this tumor were incubated with tritiated leucine or phenylalanine, the amino acids were incorporated into insulin as well as a larger molecular weight protein having a higher specific activity than insulin. A close relationship of this material to insulin was shown by its strong reaction with insulin antisera and the presence of the same N-terminal amino acid as the B chain of insulin. Incubation of this protein with low concentrations of trypsin resulted in its conversion to insulin or a closely related form, while cleavage of its disulfide bonds by sulfitolysis did not yield separate A or B chains, but indicated the presence of a polypeptide chain larger than insulin. Moreover, insulin was not released from it by extremes of pH, 8 *M* urea, or concentrated acetic acid. It was thus concluded that this protein, later named proinsulin, was a precursor of insulin and consisted of a single polypeptide chain beginning at the N-terminus with the B chain sequence of insulin, terminating with the A chain and bearing an additional polypeptide connecting the A and B chains (9). When a second insulinoma became available, the time course of incorporation of radioactive amino acids into proinsulin and insulin was studied. Radioactivity appeared first in proinsulin, and only after approximately 60 to 80 minutes of incubation was labeled insulin detectable in the tumor slices (10).

Because of the infrequent occurrence of β cell tumors in man, further studies were performed on isolated islets of Langerhans obtained from rat pancreas by collagenase digestion (10, 11). After partial purification and gel filtration two major peaks of radioactivity were seen similar to that observed in human islet tumors. Time course studies showed that most of the radioactivity was present in the proinsulin peak during the first hour. Thereafter, counts appeared at a position corresponding to insulin, and with time the insulin peak increased while that of proinsulin tended to plateau. The transfer of radioactivity behaved like a first-order process with a half-life of about one hour for proinsulin (12). Inhibition of protein synthesis with cycloheximide did not prevent the transfer of radioactivity from proinsulin to insulin.

These observations were rapidly confirmed by Tung & Yip (13) who showed that incubation of fetal bovine pancreas slices with tritiated leucine resulted in the incorporation of radioactivity into a protein which was immunologically related to insulin and could be converted to insulin by exposure to trypsin. Similar results were also obtained by Grant & Reid (14) using slices of cod islet tissue incubated with radioactive proline. Initially all the radioactivity was incorporated into a polypeptide with molecular weight of approximately 10,000, but after one hour there was a significant increase in the radioactivity of insulin. Gel filtration of the polypeptide after oxidative sulfitolysis indicated that it was a single polypeptide chain, and treatment with low concentrations of trypsin caused a progressive increase in the formation of an insulin-like material. More recently, Lazarus and coworkers (15) have identified proinsulin in a human pancreatic adenoma incubated in vitro with radioactive leucine.

The presence in the rat of two insulins differing slightly in primary structure provided an opportunity to determine whether a separate proinsulin would precede the formation of each insulin (16). By carrying out polyacrylamide gel electrophoresis at pH 4.4, two proinsulins, only one of which contained methionine which is unique to rat insulin II, were identified. Furthermore, the proinsulin fraction obtained by gel filtration contained three minor components, each of which yielded approximately equal amounts of N-terminal phenylalanine and glycine. These components were thought to represent partially cleaved two-chain intermediate forms in the conversion of proinsulin to insulin. Similar two-chain proinsulin intermediates were found as major components in the mitochondrial-granule fraction of bovine fetal pancreas (17), as well as in extracts from human β cell tumors (15) and cod islet tissue (14).

PROINSULIN BIOSYNTHESIS AND TRANSFORMATION

Site.—There are many similarities in the biosynthetic and secretory activities of pancreatic β and acinar cells. Jamieson & Palade (18, 19) have demonstrated by radioautography and pulse chase experiments that newly synthesized secretory protein is first sequestered in the cisternae of the endoplasmic reticulum and then transported through the Golgi apparatus to the site of granule formation. Insulin (proinsulin) biosynthesis has also been localized to the ribosomes of the endoplasmic reticulum (20) and subsequently a similar progression of labeled protein through subcellular fractions of the β cell has been observed (21, 22). After 10 minutes most of the radioautographic silver grains were situated over the endoplasmic reticulum and cytoplasm, while at 60 minutes a majority of the grains was associated with β granules. The Golgi apparatus was maximally labeled at 20–45 min (21). Logothetopoulos (23), Orci et al (24), and Like (25) have recently presented morphological evidence for the origin of new beta granules from the periphery of the Golgi complex. By pulse labeling isolated rat islets with leucine, Sorensen, Steffes & Lindall (26) have found that radioactive protein appeared first in the microsomal fraction. The specific activity of this fraction was more than three times greater than the mitochondrial-secretion granule fraction at 50 minutes. However, by 60 minutes the specific activity of this latter fraction had risen rapidly above that of the microsomes, indicating accumulation of newly synthesized protein in secretion granules.

Consideration of both kinetic and morphological data suggests that transformation of proinsulin to insulin occurs within the Golgi complex or during the subsequent phase of granule formation and maturation (27).

Addition of antimycin A, a potent inhibitor of oxidative metabolism, to isolated islets at intervals following brief labeling with radioactive amino acids, completely blocked proinsulin conversion to insulin (28), presumably by inhibiting the transit of newly synthesized proinsulin from the rough endoplasmic reticulum to the Golgi region. Sorensen, Steffes & Lindall (26) have shown that the product transferred from the microsomes to the secretion granule fraction of rat β cells was proinsulin, and that this latter fraction was the most active in converting proinsulin to its intermediate form and insulin. Similar results were obtained by others, the two-chain proinsulin intermediate being found in greatest amounts in the mitochondrial-granule fraction of fetal bovine pancreas (17), while the single-chain precursor was maximal in the microsomal fraction. Positive immunoreaction has been obtained in β cells of fetal and adult bovine islets with specific antiproinsulin antibody preparations, but the resolution of the technique did not permit specific localization of immunofluorescence within subcellular organelles (29).

Regulation of biosynthesis.—Raising the concentration of glucose from 1 to 5 mg/ml in the incubation medium of isolated rat islets resulted in a threefold stimulation of insulin synthesis (16), whereas raising the level from 2 to 6 mg/ml doubled the rate. In the presence of 16 m*M* glucose, dibutyryl clyclic adenosine-3′5′-monophosphate and theophylline significantly enhanced the synthesis of proinsulin and insulin (30). On the other hand, the effect of tolbutamide was variable (30, 31). Similar results were noted with rabbit islets which synthesized approximately eight times as much insulin in a medium that contained 2.5 mg/ml glucose as compared to one containing 0.5 mg/ml (32). Actinomycin D did not inhibit the stimulation of insulin biosynthesis by glucose, thus suggesting that new RNA synthesis was not required for the glucose effect (31).

Transformation to insulin.—In vitro exposure of labeled human (9) and rat (16) proinsulin to low concentrations of trypsin rapidly transformed it so that it eluted at the same position as insulin after gel filtration. With larger amounts of trypsin the elution position shifted beyond that of intact insulin to a position corresponding to desoctapeptide insulin, with release of the heptapeptide (B 23–29) of the B chain. As the product of trypsin digestion lacks threonine (human) or serine (rat) at B 30, this enzyme cannot fulfill all the requirements of the proposed intracellular converting enzyme. However, a modified trypsin-like enzyme, possibly acting in conjunction with a protease having specificity similar to carboxypeptidase B, could account for the generation of normal insulin in vivo (11). Although this enzyme or system has not yet been isolated, its activity can be partially inhibited by O-ethyl O-*p*-nitrophenyl phenylpropylphosphonate in the cod (14) and by N-ethyl maleimide in the rat (33).

Fate of the C-peptide.—In studies on the biosynthesis of insulin in isolated rat islets, the free C-peptide was detected when an appropriate precursor, such as leucine or proline, which occurs in the peptide, was used (16). The C-peptide eluted at nearly the same position as insulin from columns of Sephadex G-50, but was separated from the insulin by electrophoresis on polyacrylamide gels at pH 8.9 or by paper electrophoresis in 30 percent formic acid. The relative amount of labeled C-peptide within incubated islets remained essentially equivalent to that of labeled insulin over many hours (34). These findings suggest that after the proteolytic transformation of proinsulin to insulin, both the C-peptide and insulin are stored together presumably within secretory granules where the conversion may actually take place.

Quantitative relationship of proinsulin and insulin.—Immunoassay of column fractions from acid-ethanol-extracted rat islets (12) and whole pancreas (35) showed less than 5 percent of the total immunoreactivity in the proinsulin region. Similar relative proportions have been found in pork and ox pancreas (J. Schlichtkrull and O. Hallund, personal communication), and in human fetal pancreas irrespective of the age of the fetus (36). These ratios indicate that the pool of proinsulin is small in relation to stored insulin and are consistent with the precursor role of proinsulin. In striking contrast to these results, however, is the finding that proinsulin comprised up to 50 percent of the total "insulin" immunoreactivity extracted from a human islet cell tumor (37).

STRUCTURAL STUDIES

Isolation of proinsulin from crystalline insulin.—The stimulus leading to the isolation of proinsulin from insulin preparations differed among various laboratories. Thus, Steiner and co-workers (38), upon consideration of the similarities of proinsulin and insulin anticipated that they might crystallize together. On the other hand, Chance, Ellis & Bromer (39) while studying the properties of minor components of crystalline insulin, recognized that the properties of one of their fractions obtained after DEAE-cellulose chromatography resembled those described for proinsulin. When crystalline preparations of bovine or porcine insulin were gel-filtered on Sephadex G-50 in 1 *M* acetic acid, a small peak comprising 2–3 percent of the total protein eluted in the characteristic position of proinsulin (9, 11 12). After incubation with trypsin, approximately two-thirds of the material eluted in the position of insulin, indicating that only a portion of the fraction was similar to proinsulin.

Gel electrophoresis of this crude proinsulin fraction demonstrated three major and several minor bands (38). The slowest migrating major fraction corresponded in properties to proinsulin isolated from biosynthetic studies. It yielded only phenylalanine on N-terminal analysis and eluted as a single peak from Sephadex G-75 after oxidative sulfitolysis, thus confirming its single-chain structure. All three major protein fractions reacted well with insulin antisera, but, in contrast to the other two, the fastest moving component was not transformed to insulin by trypsin ("nonconvertible"). This fraction was found to have the same amino acid composition as bovine insulin, but did not fully dissociate into A and B chains after oxidative sulfitolysis or performic acid oxidation. It is therefore likely to be an abnormal dimer of insulin, but the nature of the aberrant linkage between the two insulin molecules is uncertain. Using similar techniques, the intermediate fraction was shown to consist of two closely related two-chain forms of proinsulin (11). Yip & Lin (40), Yip (41), and Schmidt & Arens (42) also noted the presence of similar intermediate forms in bovine material. Gel filtration of twice crystallized rat insulin separated 1.5 percent of higher molecular weight material which consisted of predominantly single-chain proinsulin and the intermediate component (16). However, possibly because of the long time interval between death and postmortem examination, the crude proinsulin fraction obtained from human insulin crystals consisted mainly of material corresponding to the nonconvertible fraction (43, 44).

Structure of proinsulin.—Chance, Ellis & Bromer (39) isolated porcine proinsulin during the chromatography of porcine insulin on columns of DEAE-cellulose in urea containing buffers. The molecule consisted of 84 amino acids with phenylalanine as the sole N-terminal residue and asparagine as the single carboxyl amino acid (39, 45). Electrophoresis on both cellulose acetate and polyacrylamide gels after conversion to either the corresponding S-sulfonate or reduced carboxymethylated form confirmed its single-chain structure. The amino acid sequences of both porcine and bovine proinsulin have been elucidated (11, 39). Both molecules are ordered as: NH_2-B chain. Arg. Arg. C-peptide. Lys. Arg. A chain-COOH. "C-peptide" thus refers to the connecting segment aside from the terminal pairs of basic residues. The connecting segment of bovine proinsulin (F.W.-8684) is 3 residues shorter than the 33 residue connecting segment of porcine proinsulin (F.W.-9082) and the two sequences differ by about 30 percent (11, 39). However, the basic residues attached to either end of the C-peptide are the same in the two species (Arg-Arg at the amino terminus and Lys-Arg at the carboxyl terminus of the connecting segments) in keeping with their presumed function as sites of enzymatic cleavage in the conversion of proinsulin to insulin. The intermediate fraction consists of two closely related forms (11, 28), one of which has been cleaved in the carboxyl terminal region of the connecting segment with elimination of lysine (residue 59) and arginine (residue 60). The other has been cleaved in the amino terminal region of the connecting segment with loss of the Arg-Arg dipeptide (residues 31 and 32). Although it is not certain whether these forms are true physiologic intermediates corresponding to the labeled two-chain forms of proinsulin isolated in biosynthetic experiments (16, 17), the available evidence favors this possibility. The compositional study of proinsulin reported by Yip & Lin (40), was carried out on the crude proinsulin fraction from gel filtration and thus represented the composition of a mixture of several components (41).

The amino acid compositions of cod (14), angler fish (46), and human proinsulin (47) have been published. Their connecting polypeptide segments contain 30, 33, and 35 amino acids, respectively, and in addition show many differences in composition.

Physical properties of proinsulin.—Sedimentation equilibrium studies confirmed the molecular weight of 9100 for porcine proinsulin (48), which is similar to the values estimated for bovine proinsulin (38, 49). Proinsulin and insulin have similar self-association characteristics and ultraviolet absorption spectra, thus leading Frank & Veros (48) to propose that the insulin moiety of proinsulin exists in the same conformation as in the native molecule. Proinsulin also interacts with zinc in a 3 to 1 molar ratio similarly to insulin (50). Although the optical rotatory dispersion and circular dichroic spectra of proinsulin indicated some differences in α-helical content compared to insulin, it could be accounted for by assuming that the connecting segment exists in a random coil formation (48).

Isolation of the C-peptide.—Evidence from biosynthetic experiments indicated that following the transformation of proinsulin, the C-peptide was retained within the β cells together with insulin (34). It thus seemed probable that the C-peptide would be found in normal pancreas in amounts correspondent with insulin on a molar basis. Clark et al (51) and Steiner et al (52a) extracted bovine, human, and monkey pancreas and separated the fractions containing both insulin and C-peptide by gel filtration. This material was then subjected to gradient elution chromatography on carboxymethyl cellulose and the C-peptide was further purified by paper electrophoresis and partition chromatography on a column of formylated cellulose. Sundby & Markussen (53), using side fractions from the commercial production of insulin, purified bovine and porcine C-peptide by trichloracetic acid precipitation and ion exchange chromatography. The bovine pancreatic peptide showed identity with the C-peptide derived from the connecting segment of bovine proinsulin (or the intermediate forms) with respect to its exclusion volume on gel filtration, electrophoretic mobility, Rf on thin layer chromatography and immunological properties. Moreover, amino acid sequence analysis of the peptide and its chymotryptic fragments demonstrated its structural identity with the authentic proinsulin C-peptide. This evidence strongly supports the view that the pancreatic C-peptide is derived from proinsulin. Equimolar amounts of C-peptide and insulin were recovered from fresh bovine pancreas, but owing to extensive autolysis in human pancreas obtained at autopsy, the yields of C-peptide were considerably lower (52a, b). The ready availability of the more abundant C-peptide opens up the possibility of determining its amino acid sequence in a wide range of species.

Structure of human C-peptide.—Human C-peptide contains 31 amino acids and its sequence, which differs considerably from bovine and porcine C-peptide, has been elucidated (52b, 54). Its composition corresponds well to the residues present in the connecting segment of human proinsulin, apart from the four additional basic amino acids in the latter protein.

ROLE OF PROINSULIN IN BIOSYNTHESIS

The precise role of the connecting segment in the proinsulin molecule is still open to speculation. However, it appears that its major function is to facilitate the formation of the native structure of the insulin molecule by ensuring the correct pairing of cysteine residues for the formation of the interchain disulfide bonds of insulin during biosynthesis. This hypothesis has been strengthened by the demonstration that after reduction in 8 *M* urea, reoxidation of bovine and rat proinsulins (55) result in the regeneration of 60–70 percent of their immunological reactivity, whereas insulin and the two chain intermediate forms do not refold to greater than 1–2 percent. The reoxidized bovine proinsulin behaved similarly to the native protein on gel filtration and polyacrylamide gel electrophoresis, and was converted quantitatively to desalanine insulin by incubation with trypsin. The desalanine insulin gave a correct amino acid analysis after crystallization and was fully active both biologically and immunologically. The restoration of these properties strongly suggested that the disulfide bonds of the reoxidized proinsulin were identical to that of native proinsulin and insulin.

Although the sequences of bovine, porcine and human C-peptide vary considerably, certain properties are well conserved. The terminal residues of the peptides are identical and acidic residues are conserved both with respect to number and position. Aromatic residues, histidine and sulfur-containing amino acids are uniformly absent, and at either end of the connecting peptide are regions having a high proportion of polar residues (52a, b). The central part of the molecule contains a high concentration of glycine surrounded by nonpolar residues. Thus, it is probable that the C-peptide not only converts the bimolecular reaction of chain recombination to a unimolecular reaction, but does contain certain specific structural characteristics necessary for efficient peptide folding.

IMMUNOLOGICAL PROPERTIES

Preparation of labeled proinsulin and C-peptide.—Human, porcine, and bovine proinsulin (44, 56) have been labeled with ^{125}I and ^{131}I to specific activities of 100–500 mC/mg without difficulty. The integrity of the labeled protein was shown by the quantitative conversion of radioiodinated proinsulin to material behaving like desalanine insulin following incubation with trypsin, and the demonstration of a single radioactive peak both before and after oxidative sulfitolysis. In order to facilitate the iodination of bovine, porcine, and human C-peptides, which do not contain tyrosine, this amino acid has been coupled to the peptides using N-carboxytyrosyl anhydride (57).

IMMUNOLOGICAL REACTIONS OF PROINSULIN, INSULIN, INTERMEDIATE FORMS, AND C-PEPTIDE

With insulin antisera.—Although most insulin antisera react less well with proinsulin than with insulin, there is wide variation in the degree of crossreactivity (56–61). The bovine intermediate forms and the nonconvertible fraction have greater reactivity than proinsulin (56), but the C-peptide does not compete with a tracer of ^{131}I-insulin in this system (57, 60). It seems probable that the proinsulin-connecting segment interferes with the access of some insulin antibodies to the insulin moiety of the proinsulin molecule, or that the attachment of the connecting segment to the ends of the A and B chains decreases the affinity of antibodies directed against these two specific areas of the insulin molecule.

With proinsulin antisera.—Antisera prepared against purified bovine proinsulin discriminated well between proinsulin and its intermediate forms on the one hand and insulin and the nonconvertible fraction on the other (56). The presence of antibodies directed specifically against the connecting segment was demonstrated by the inability of large quantities of insulin to compete with ^{131}I-proinsulin for all the binding sites of the antiserum (56), and by the affinity of the antiserum for ^{131}I-tyroslyated C-peptide (57). Although its reactivity varied with different antisera, the free C-peptide decreased the ^{131}I-proinsulin bound to proinsulin antisera (56, 57, 62).

As the cross-reaction with insulin in certain antisera was weak, preincubation of these proinsulin antisera with insulin resulted in a system which selectively measured proinsulin and was not influenced by added insulin (56, 62). Yip & Logothetopoulos (63) and Root and co-workers (64) adsorbed their proinsulin antisera with an immunoadsorbent of insulin conjugated to Sephadex and succeeded in removing those antibodies which cross-react with insulin. The resulting antiserum was highly specific for proinsulin and the C-peptide.

With C-peptide antisera.—As repeated immunization of guinea pigs with C-peptide alone did not result in the production of antibodies, human C-peptide was coupled to rabbit albumin with 1-ethyl-3-(3-dimethyl aminopropyl carbodiimide hydrochloride). The resulting antiserum reacted with human proinsulin and C-peptide, but not with insulin (47). The reduced affinity of proinsulin compared to C-peptide in this system was presumably due to the additional presence in proinsulin of the four basic connecting residues and the insulin moiety. These may either alter the tertiary configuration of the peptide or sterically hinder certain of its antigenic determinants.

COMPARATIVE ASPECTS OF BOVINE, PORCINE, AND HUMAN PROINSULIN AND C-PEPTIDE

Using porcine or bovine proinsulin antisera, heterologous proinsulins reacted similarly to equimolar concentrations of insulin, while increasing concentrations of the homologous proinsulin competed more effectively with the tracer to give a steep slope (57). Removal of the insulin-reactive antibodies from bovine and porcine proinsulin antisera abolished their capacity to react with heterologous proinsulins as well as insulin (63, 64). Using bovine proinsulin antiserum and ^{131}I-tyrosylated-C-peptide, the human and porcine C-peptide reacted 250 and 750 times less efficiently, respectively, than the bovine molecule (57). Similar results were obtained with human C-peptide antiserum, for only human proinsulin and C-peptide and the closely related monkey C-peptide competed with ^{131}I-tyroslyated human C-peptide for binding sites (47). These findings are not surprising in view of the marked differences in structure of the connecting regions of proinsulin, and indicate that specific antisera to each will be needed for optimal assay specificity.

SECRETION AND CIRCULATION OF PROINSULIN AND C-PEPTIDE

SECRETION OF PROINSULIN AND C-PEPTIDE IN VITRO

Proinsulin.—Although glucose results in a rapid release of insulin from incubated islets, newly synthesized insulin is secreted only after about 1 hour (65). After 65 minutes of incubation in medium containing 3 mg/ml glucose, only 1 percent of the total labeled acid/alcohol-soluble protein of the islets was found in the medium, while at 200 minutes, there was a significant rise to 10 percent (12). The largest increment of radioactive insulin appeared in the medium between 120 and 150 minutes, at which time there was a declining rate of insulin secretion (26). Higher glucose concentrations in the bathing medium not only stimulated the biosynthesis of the hormone, but enhanced the secretion of newly synthesized insulin (16, 30). An increase in glucose concentration from 2 to 6 mg/ml resulted in a 1.5-fold increase in insulin secretion during a 4.5-hour incubation (16).

Proinsulin and its intermediate fractions have also been identified as secretion products of isolated rat islets (16, 30). A series of control experiments have clearly demonstrated that the appearance of proinsulin in the medium represents release and not leakage from damaged islets (30). Proinsulin secretion was stimulated by an increase in glucose concentration and by theophylline, tolbutamide, and dibutyryl cyclic adenosine-3′5′-monophosphate in the presence of glucose (16, 30). These agents increased the medium to islet ratios for both proinsulin and insulin, as well as the relative proportion of proinsulin/insulin in the medium (30). Essentially similar results were obtained using a human islet cell tumor (15). Burr and co-workers (35) have measured the fraction of total immunoreactive insulin activity which is represented by proinsulin in the perfusate from pieces of rat pancreas. In the perfusate collected after 55–60 minutes of stimulation with glucose, the percentage of proinsulin had reached 17 percent, a tenfold rise from prestimulation levels.

C-peptide.—Gel filtration and polyacrylamide gel electrophoresis of acid-ethanol extracts of medium from isolated rat islet incubation have also identified the C-peptide as a secretion product of the β cell. The labeled C-peptide was liberated simultaneously with newly synthesized insulin in equimolar amounts (34). These findings are consistent with the packaging and retention of the C-peptide with insulin in secretory granules after the transformation of proinsulin.

SECRETION OF PROINSULIN AND C-PEPTIDE IN VIVO

Proinsulin.—When samples of human serum and urine were gel filtered, two peaks of immunologically reactive material were found (58, 66). The earlier one corresponded in position to proinsulin and the second to insulin. Although Roth, Gorden & Pastan (58) named the higher molecular weight material "big" insulin, it is now generally accepted by them (67) and others (15, 45) that it represents proinsulin and/or its intermediate forms. Until methods are devised to separate and identify these different proteins in plasma, it would seem advisable to refer to them as proinsulin-like components (PLC). In assays using porcine ^{131}I-insulin and an antiserum against porcine insulin, serial dilutions of serum PLC showed immunological identity with a human proinsulin standard (68). The strong reaction of the early eluting plasma component with an antiserum to human C-peptide confirmed its identity inasmuch as antigenic determinants of the connecting polypeptide segment must be present in order for an immunological reaction to occur with this system (47). Tryptic digestion of serum PLC converted it to products which were indistinguishable from insulin and desoctapeptide insulin (68, 69). PLC has also been identified after gel filtration of bovine serum using highly specific bovine proinsulin antisera (34, 63), and its pancreatic origin in the dog was confirmed by demonstrating higher levels in the pancreatic vein than in peripheral arterial blood (58).

The fasting values of PLC in normal subjects ranged between 0.05–0.4 ng/ml representing from 5–48 percent of the insulin concentration (68). Since proinsulin constitutes only 1 to 2 percent of the total insulin in the pancreas, these values suggest that a significant portion of basal circulating immunoreactive insulin may be newly synthesized hormone rather than stored insulin from granules (67). This may imply a different mode of secretion in the fasting state, in keeping with observations of others that the regulation of basal insulin secretion may differ from that following various stimuli (70–72). As yet, the significance of the high basal secretion of proinsulin is unclear, but should provide a fruitful area for future investigation.

After oral administration of glucose the levels of PLC and insulin both increased. However, the rise in PLC occurred later and, in some subjects, was of lesser magnitude than insulin. When the values for serum PLC were expressed as percentage of the insulin levels, there was a decrease in the first 15–60 minutes of the glucose tolerance test. Thereafter proinsulin contributed an increasing amount to the immunoreactive insulin level (68). A similar pattern was observed following the administration of arginine and tolbutamide (73). An enhancement of PLC levels at 2 hours was noted in healthy subjects who were given glucose after 48 hours of starvation (73). The mean excretion of urinary PLC during 24 hours in healthy subjects was 45.8 ng and the proinsulin: insulin ratio 13.7 percent (74).

Obese patients with hyperinsulinemia had higher fasting levels of proinsulin and a greater rise after glucose than normal subjects (68). As the high levels of proinsulin coexisted with raised insulin concentrations, the relative proportions of the two hormones were generally in the same range as observed in normal subjects. However, Gorden & Roth (69, 73) have suggested that the proportion of PLC might be higher in obese subjects than in controls 15–30 minutes after oral glucose, and that this elevation in PLC was roughly proportional to their increase in percentage of ideal body weight and elevation in fasting plasma insulin concentrations. Similar results were found in acromegalics with raised fasting plasma insulin levels (73). Three young thin patients with idiopathic glucose intolerance and patients with myotonic dystrophy had PLC responses which were indistinguishable from those in normal subjects (69). Goldsmith, Yalow & Berson found that the serum PLC comprised less than 20 percent of the total immunoreactive insulin in 55 samples from a variety of patients, many of whom showed glucose intolerance and insulin insensitivity (59). However, it should be noted that plasma samples with high insulin levels obtained after glucose, tolbutamide, arginine, or glucagon stimulation were specifically chosen.

Serum PLC has also been measured in a number of patients with islet cell tumors (15, 37, 59, 73). Two patients with metastatic islet cell carcinomas had 18 and 37 percent PLC after fasting 200 minutes (73). Goldsmith, Yalow & Berson (59) found values of PLC between 24–55 percent of the insulin immunoreactivity in a patient with an adenoma, while a single sample from another such patient showed only 5 percent. Relative PLC concentrations of greater than 50 percent have been reported by Lazarus and coworkers (15) and Melani et al (37), as well as an increase in the absolute PLC level after tolbutamide. In the patient described by the latter group, PLC represented only 10 percent of total serum immunological activity after removal of the adenoma. Serial dilution of serum from an insulinoma patient resulted in nonlinear increases in immunoreactive insulin, which has been attributed to the presence of high levels of proinsulin (75). The presence of high levels of circulating proinsulin in these patients might be the result of a relative or absolute deficiency of the converting enzyme or defective storage mechanisms in such tumors.

C-peptide.—C-peptide was first detected in extracts of bovine plasma obtained from cows in the fasting state and 1 hour after a glucose load (34). After gel filtration, the fractions corresponding to insulin or proinsulin markers were tested in three assay systems: insulin antiserum and ^{131}I-insulin; proinsulin antiserum and ^{131}I-proinsulin; and proinsulin antiserum preincubated for 24 hours with insulin and ^{131}I-proinsulin. Both insulin and proinsulin were detected in the insulin and proinsulin assays. On the other hand, although insulin did not react in the preincubated assay, immunologically reactive material corresponding to standards of C-peptide was identified in the insulin region of the column. The level of C-peptide was higher in the 60-minute sample suggesting that it may be secreted in response to glucose.

Following the development of an assay for human C-peptide, this peptide was identified in extracts of human serum after gel filtration (47). The levels of C-peptide in each sample corresponded closely on a molar basis to those of insulin, suggesting that these substances are secreted in essentially equivalent amounts during emiocytosis of beta cell granules. The presence of C-peptide in serum in addition to proinsulin precludes the direct measurement of these peptides in unextracted samples, for most proinsulin and C-peptide antisera react significantly with both polypeptides.

PERIPHERAL METABOLISM

The immunological half-life of porcine proinsulin was 22 minutes in pigs and 18 minutes in baboons, about twice that of insulin (62). Similar values were found after the injection of bovine proinsulin in rats and dogs (unpublished observations). In contrast to insulin, proinsulin and C-peptide were not cleared by the isolated perfused rat liver (62, 76). However, considerable proinsulin (and insulin) was extracted by the dog kidney, the renal arteriovenous concentration difference being 23–50 percent of the prevailing arterial proinsulin concentration (unpublished observations).

Labeled proinsulin has been injected intravenously and subcutaneously into rats to determine whether it is converted to intermediate forms or insulin-like components in the circulation (43, 77–79). However, no transformation has been observed as late as 60 minutes after intravenous or 3 hours after subcutaneous injection. Gel filtration of acid-ethanol extracts of liver and kidney of rats injected 20 and 60 minutes earlier with labeled proinsulin have also not shown transformation of proinsulin to insulin in either tissue. Similar results were obtained with rat hemidiaphragms (77), the radioactive proinsulin in the incubation fluid retaining its characteristics on gel filtration and polyacrylamide gel electrophoresis. Nevertheless, on the basis of immunological measurements, Mashiter & King (80) have suggested that a number of rat tissue extracts can convert proinsulin to insulin, the liver being most active. It is obvious that the entire question of the peripheral metabolism of proinsulin and its relationship to biological activity needs critical re-examination before definitive answers will be forthcoming.

BIOLOGICAL ACTIVITY

There has been considerable interest in the biological activity of proinsulin. However, few authors have interpreted their results in the light of the absolute levels of circulating proinsulin and the possible biological effect of these relatively low concentrations of hormone on peripheral tissues. Furthermore, most studies have been concerned with the activity of proinsulin on isolated tissues in vitro, a situation that may differ considerably from that pertaining in vivo. Nevertheless, the widely divergent, unexpected and unexplained findings have stimulated a great deal of interest in the mechanism whereby proinsulin exerts its biological activity, including its possible extra-pancreatic conversion to insulin and interaction with insulin at peripheral receptor sites.

Proinsulin.—The biological potency of proinsulin in the intact animal is still open to question. Chance, Ellis & Bromer (39) have reported that purified porcine proinsulin exhibited insulin-like activity to the extent of 3 I.U./mg by the mouse convulsion assay. On the other hand, purified bovine proinsulin gave values of approximately 10 I.U./mg when measured by its effect on the blood sugar level of fasted rats (81), but had relatively less effect in fed rats and fasted mice (81). Lazarus and co-workers injected porcine proinsulin into fasted rats and found that it was at least four times less active than insulin on a molar basis (77). Hypophysectomy and adrenalectomy increased the hypoglycemic response of rats to proinsulin (77), whereas pancreatectomized, nephrectomized, and two-thirds hepatectomized rats responded to proinsulin similarly to normal animals (81). Compared to insulin, proinsulin resulted in a more protracted decrease in blood sugar in both animals and man (81, 82). The relationship of this latter effect to the longer half-life of proinsulin in the circulation needs further study.

Although Willms et al (83) have claimed that eviscerated, hepatectomized rats lose their hypoglycemic responsiveness to proinsulin, this has been denied by others (84). Proinsulin was less active than insulin when infused into the forearm of human subjects, but its effects upon adipose tissue were relatively greater than upon muscle (85).

The activity of porcine and bovine proinsulin on rat diaphragm muscle was approximately 20–30 percent that of insulin when compared on a molar basis (77, 86). However, the maximal stimulatory effects of proinsulin and insulin were similar, and both stimulated the uptake of glucose and formation of glycogen. Proinsulin influenced the same metabolic functions as insulin in epididymal fat tissue and isolated fat cells, increasing glucose uptake from the medium, its oxidation to carbon dioxide and incorporation into triglyceride, and decreasing lipolysis induced by epinephrine, theophylline, and glucagon. Although proinsulin consistently showed lower biological activity than insulin, there was great variability in their relative potencies in fat cells compared to fat tissue, and when different metabolic parameters were measured in either system. Thus, proinsulin has been described as having 10–18 times less activity than insulin with respect to glucose oxidation in fat tissue (86, 87), and 9 times less antilipolytic activity (87). Comparable experiments on fat cells showed proinsulin to be 10–50 times less effective than insulin in stimulating glucose oxidation (60, 87, 88), and 30–90 times less in antilipolytic activity (87–89).

The inhibitory effect of Kunitz pancreatic trypsin inhibitor (KPTI) on some, but not all, the biological actions of proinsulin is intriguing. Shaw & Chance (86) hypothesized that proinsulin had very low intrinsic biological activity, but was activated by conversion to insulin in peripheral tissues. KPTI was used in the hope of preventing this proteolytic conversion process in fat and muscle cells or their interstitial tissue. However, as yet peripheral transformation of proinsulin has not been demonstrated and the mechanism of action of KPTI is unclear. KPTI blocks the stimulatory effect of proinsulin on glycogen formation and glucose transport in rat hemidiaphragm (77, 86), and glucose oxidation in epididymal fat tissue (86). It does not inhibit proinsulin's antilipolytic effect on fat pieces (89) or cells (87–89), nor its stimulation of glucose oxidation in fat cells (60, 88). Furthermore, injection of KPTI had no effect on proinsulin-induced hypoglycemia in rats (77). It remains to be determined whether these peripheral actions of proinsulin are significant under physiological circumstances.

C-peptide.—There is little information regarding the extrapancreatic functions of the C-peptide. Preliminary studies have shown no effects of this peptide on several parameters of insulin action in vitro (60). However, these studies have been carried out with bovine or porcine C-peptide in rat tissue. Due to the large sequence variation among species, it will be important to carry out studies of the peripheral activity of the C-peptide in the homologous species.

CONCLUSIONS

The recent discovery of proinsulin stimulated the interest of investigators in such diverse fields as protein chemistry and biosynthesis of peptide hormones, regulation of carbohydrate metabolism, and mechanisms of hormone action. More particularly, the existence of proinsulin and an enzymic system necessary for its conversion to insulin raises new possibilities in the search for genetically determined abnormalities which could account for inherited diabetes. Mutations in the connecting segment of proinsulin might impair the efficiency of folding of proinsulin or interfere with its conversion to insulin. Similarly, abnormalities in the proinsulin-converting enzymes might limit the capacity of the pancreas to produce biologically active insulin. Identification of proinsulin in the circulation has encouraged the study of other hormones in blood with similar fractionation techniques, and preliminary results with gastrin, glucagon, growth hormone, and vasopressin have proved equally exciting. The extrapancreatic role of the C-peptide and proinsulin is still unclear, and the relatively high proportion of proinsulin in fasting sera needs further evaluation. Finally, the commercial production of highly purified insulin for therapeutic purposes is now underway, and the possible beneficial effects of treating diabetic patients with this product will be studied.

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MALABSORPTION DUE TO LYMPHOMATOUS DISEASE

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INTRODUCTION

Lymphoma of the small intestine can occur as a late, secondary manifestation of disseminated lymphoma or, far less commonly, as a primary lesion, originating in the small intestine or its mesenteric nodes (1–5).

The latter disease can be further subdivided into two categories: (*a*) solitary lymphoma, occurring most commonly in the terminal ileum and encountered most often in young children; (*b*) diffuse or multifocal small intestinal lymphoma, occurring most commonly in the proximal small intestine and encountered primarily in older age groups. The last of these entities differs from the other two in several important respects—most notably in the presence of malabsorption (3–5). This hallmark of diffuse primary small intestinal lymphoma tends to dominate the clinical course until its terminal stage and may in fact antedate all manifestations of neoplasia by months or years. The latter phenomenon has led inevitably to speculations concerning the existence of antecedent predisposing disorders of the small intestine or of its associated lymphoid elements. Indeed, an impressive number of cases have reportedly occurred in the course of well-documented celiac sprue, although many others have failed to exhibit evidence discernible by ordinary light microscopy of any pre-existing small bowel disease (6–11).

The syndrome of intestinal lymphoma with malabsorption, as rigorously defined by Eidelman et al in 1964 (9), occurs only rarely in the highly developed countries of the West.[1] Reviewing the medical literature of the West, those authors were able to find only 48 cases (of which they regarded 32 as well-established and 16 as probable). On the other hand, the syndrome is not at all rare among certain populations whose common denominator appears to be that they are (or were until recently) underprivileged in terms of nutrition, housing, hygiene, and medical care. In Israel, for example, the syndrome is relatively prevalent among Arabs and among first and second generation Jewish immigrants from the Middle-East and North Africa, but it is virtually nonexistent among Jews of European origin (12–14). It also appears to be relatively common among several other widely disparate populations, such as the indigenous populations of Iran (15) and of Mexico (16), and the Cape colored population of South Africa (17).

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[1] Western Europe, British Isles, United States of America, and Canada are referred to hereafter as The West or western countries.

The evidence thus far would seem to suggest that predisposition to primary small intestinal lymphoma is environmentally determined. One may also speculate that the unique vulnerability of certain populations is attributable to genetic factors, or to a combination of environmental and hereditary influences.

The present review concerns a few of the clinical and pathological aspects of primary small intestinal lymphoma with malabsorption, the possible relationship of the syndrome to pre-existing disease of the small bowel or of the reticuloendothelial system, and some of the evidence implicating environmental and genetic factors in the genesis of the disease.

CLINICAL FEATURES OF PRIMARY INTESTINAL LYMPHOMA IN WESTERN COUNTRIES

Intestinal lymphoma (or "Reticulosis") has been accepted as a rare cause of malabsorption ever since the association was pointed out by Fairley & MacKie in 1937 (18). In 1953, the topic was reconsidered by Sleisenger et al who presented three cases of their own, and the cumulative literature on the subject was comprehensively reviewed in 1964 (3, 9). Patients exhibiting the syndrome were a clinically homogeneous group. The great majority were over 50 years of age. The male:female ratio was about 3:1. The history of malabsorption varied from several months to many years and was eventually verified by laboratory data in many cases. In seven patients, peroral biopsy of the small intestine revealed the characteristic lesion of celiac sprue. Peripheral lymphadenopathy was observed in only one patient and splenomegaly in two. An abdominal mass was rarely palpated.

Two additional series, as well as individual case reports, have since been published (10, 11, 19) and have conformed to the clinical pattern alluded to above. A long history of malabsorption was elicited in a number of instances, and had been present since childhood in two cases. Features attributable to lymphoma included prolonged fever, skin rashes, and abdominal masses. Peroral biopsy frequently exhibited changes in villus architecture and in some cases these were considered indistinguishable from the lesion of celiac sprue (10, 11, 20). Some of the patients responded temporarily to a gluten-free diet (9, 11). The foregoing findings suggest rather strongly that primary small intestinal lymphoma, in western countries at least, is intimately related to celiac sprue. This impression is strikingly reinforced by a recent retrospective study of 202 patients with celiac sprue, followed for an average of 8.2 years (21). Fourteen of these patients developed intestinal lymphoma and 13 others were stricken with carcinomata of the gastrointestinal tract (including six esophageal carcinomas), clearly suggesting that celiac sprue is related in some way to neoplasia in general and to lymphoma in particular. Benson et al (22) found an almost identical incidence of intestinal lymphoma, albeit in a much smaller sample of patients.

This remarkably high incidence of malignancy is admittedly without precedent in other studies of adult celiac disease. It remains to be seen whether this difference will prove to be attributable to shorter or less complete follow-up in the older series.

Pathology of the Lesions

A number of characteristic pathologic features can be gleaned from the published series. Distribution of the disease can be in the form of a solitary mass, multiple masses, or a diffuse infiltration of the small bowel, and there is a high incidence of jejunal involvement. The abdominal nodes are usually involved. In some patients the main pathologic changes are in the mesenteric lymph nodes and very little is found in the gut.

The relative frequency of histologic tumor-types varies from one series to the next. (9–11). All in all, the most common histologic diagnosis was reticulum cell sarcoma. In 2 of 13 patients described by Brunt (11), the diagnosis was extramedullary plasmacytoma.

Malabsorption and Lymphoreticular Dysfunction

As already stated, a non-neoplastic malabsorptive disorder seems, in many instances, to have preceded the development of primary small intestinal lymphoma (6–8, 11, 21, 22). The former has on occasion proven to be celiac sprue (21, 22). There are shreds of evidence that celiac sprue in turn may be associated with lymphoreticular dysfunction and that the latter may accompany other malabsorptive disorders of the small intestine (10, 23). It is pertinent to review this evidence for whatever clues it may provide to the pathogenesis of small intestinal lymphoma.

Robb-Smith, in 1938, described a disease entity characterized by progressive hyperplasia of the abdominal lymph nodes and termed it "prohistiocytic fibrillary reticulosis" (24). The same author, in 1964, pointed out the association of this entity with steatorrhea and its pathologic similarity to lymphosarcoma (25). He suggested that the hyperplasia might be secondary to an autoimmune process in the gut that terminates in malignancy, in a manner analogous to the reticulosis complicating Hashimoto's thyroiditis (26). The finding of abdominal lymphadenopathy in association with a sprue-like syndrome has been described by others as well (22, 27). These authors also stressed the progressive nature of the lymphoid hyperplasia and its possible evolution to malignancy.

Celiac sprue has been found to be associated on occasion with splenic atrophy (23, 28). McCarthy et al summarized 28 cases of the latter association. The diagnosis could readily be inferred by finding Howell-Jolly bodies in the peripheral blood smear. The degree of splenic atrophy was not related to the severity of malabsorption. The pathogenesis of the splenic atrophy under these circumstances is not clearly understood. It may conceivably be a consequence of folic acid deficiency (a frequent complication of celiac sprue), since splenic atrophy is observed in folate-deficient animals and can be reversed by folate administration (29). This hypothesis deserves further study in humans.

Splenic atrophy, in turn, may bear a biologically significant relationship to lymphoreticular dysfunction on the one hand, and to intestinal lymphoma

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on the other. Small spleens have been observed in a number of patients with intestinal reticulosis (10, 11). Moreover, primary small intestinal lymphoma is usually unaccompanied by splenomegaly, even when the abdominal lymph nodes are extensively involved (9, 10, 11). Finally, a malabsorption syndrome resembling nontropical sprue has been described in dogs (30). In one such case, a lymphoma of the small intestine was found at autopsy, in association with a fibrotic spleen.

PRIMARY SMALL INTESTINAL LYMPHOMA IN ISRAEL

In Israel, as previously mentioned, the syndrome of primary small intestinal lymphoma with malabsorption is relatively common among Arabs and among Jews of Middle-Eastern and North-African origin, and is correspondingly rare among Jews of European origin (12–14). There are recent indications that this particular ethnic distribution may obtain outside Israel as well (15–17, 31). Moreover, it seems to hold true not only for primary small intestinal lymphoma, but for abdominal localization of lymphoma in general (12, 14, 32).

Primary small intestinal lymphoma, as encountered in Israel, differs in several other important respects from its counterpart in Western countries (9–11, 13, 14): (*a*) it affects primarily teen-agers and young adults (onset at mean age of about 20), while most of the cases in the West have been among elderly people (onset at mean age of about 50). (*b*) The male:female ratio is 1.2:1, in contrast to a ratio of about 3:1 in the West. (*c*) It involves the duodenum and proximal jejunum with striking frequency (in contrast to primarily mid-jejunal involvement in western countries). (*d*) The usual absence of obvious mucosal pathology in intestinal segments uninvolved by tumor, in contrast to the severe abnormalities in villus architecture is alleged to occur very frequently in several Western series (9–11). It should be noted, however, that a systematic, multiple-biopsy study of uninvolved segments has yet to be performed in any large series of cases, either in Israel or in the West. The most that can be said with certainty is that in almost all the cases in Israel, concurrent celiac sprue could be confidently ruled out on a histologic basis, even when inspection was confined to portions of the gut involved by tumor (33, 34). (*e*) In Israel (as detailed below) the tumor could be classified as an "extramedullary plasmacytoma" in a relatively high proportion of cases (about 25 percent), in contrast to the apparent rarity of this type of infiltration in the western series. (*f*) The possible regional specificity of a concomitant immunoglobulin A paraproteinemia (see below).

Seligmann et al (35) have recently reported a paraproteinemia consisting of a heavy chain of IgA (probably the FC fragment) in four patients of North African and Asian ancestry with the syndrome of primary small intestinal lymphoma with malabsorption. Three additional cases of this association have been found in Israel (36, 37). The clinical features of the disease and age distribution in these seven patients did not differ from cases without this protein abnormality.

The pathology of primary small intestinal lymphoma in Israel has recently been re-evaluated in a retrospective manner (38). The gross features have been found to fall into four categories:

1. Diffuse, transmural involvement of the small intestine.
2. Small uniform nodular involvement.
3. Extrinsic mass of nodes, with infiltration toward the lumen from without.
4. Large single or multiple intraluminal masses.

Histologically, six patients, including the two with IgA paraproteinemia, exhibited a diffuse plasma cell infiltration of the gut and of the mesenteric nodes. The same histologic pattern was noted in the four patients with IgA paraproteinemia described by Seligmann et al, as well as in two of the patients described by Brunt (11, 38). No protein studies are reported in the latter instance. The final histologic diagnoses in the remaining cases reviewed were: malignant lymphoma, histiocytic type; malignant lymphoma, unclassified; and Hodgkin's disease. These findings reveal, incidentally, that the syndrome we have been considering is a pathologically heterogeneous disorder—in notable contrast to one other type of lymphoma whose occurrence seems strongly conditioned by environmental factors, namely, central African lymphoma (39).

CONCLUDING REMARKS

Any understanding of the pathogenesis of primary small intestinal lymphoma would seem to be predicated on the answers to two major questions:

A. Is the disease in fact preceded, in all or most instances, by some non-neoplastic malabsorptive disorder of the small intestine or its associated lymphoid elements.?
B. Is the antecedent disorder, or the lymphoma itself, traceable to specific etiologic factors, be they environmental or hereditary?

Preliminary answers to these questions might be provided by the following lines of investigation: (*a*) a systematic multiple-biopsy study of uninvolved portions of the small intestine in patients with primary small intestinal lymphoma; (*b*) Prospective, serial biopsy studies of patients with celiac sprue and of patients with unclassifiable malabsorptive disorders—with due attention to the range of histologic normalcy among presumably healthy adults in underprivileged populations (9, 40). (*c*) Study of all patients alluded to above by hitherto unexploited modalities, including electron microscopy (viral particles?); systematic microbiological studies of intestinal contents; determination of antibodies to common and rare antigens in intestinal secretions and in intestinal lymphoid tissue; detection of aberrations in circulating immunoglobulins of intestinal lymphoid origin. (*d*) Familial studies of patients with intestinal lymphoma and other malabsorptive disorders.

The Middle East may prove to be fertile ground for studies such as these—not only because intestinal lymphoma is endemic in that locality, but also because of a notably high prevalence of small bowel disease in general. In Israel, for example, some of the same ethnic groups that are susceptible to intestinal lymphoma exhibit a high incidence of celiac sprue, congenital B_{12} malabsorption, and unclassifiable malabsorptive intestinal disorders in general (41, 42). Indeed, the moral of our present tale may prove to lie in this very combination of circumstances.

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SUPERINFECTION IN LYMPHORETICULAR DISEASES[1]

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INTRODUCTION

During the last two decades there has been an increasing appreciation of the lymphoreticular tissues as mediators of the host's immunologic defenses. Some lymphoreticular tissues appear to mediate cellular immunity and others humoral immunity, but the precise protective function of these defenses is poorly understood. William Harvey noted that there is no

> better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of nature by careful investigation of cases of rarer forms of disease. For it has been found, in almost all things, that what they contain of useful or applicable is hardly perceived unless we are deprived of them, or they become deranged in some way (1).

In certain diseases of the lymphoreticular system the patient is deprived of either cellular or humoral immunologic defense systems, or both. In these diseases death frequently occurs not from the disease per se, but from the superinfection resulting from defective host defenses. This discussion will be limited to superinfection in those lymphoreticular diseases with detectable immunologic deficits. It is hoped that by correlating the cell type involved in the lymphoreticular disease with the nature of impairment of host defense and the usual infecting pathogens we can learn more about the protective role immunologic defenses normally play, and can develop an approach to these diseases which will help advance the "proper practice of medicine."

DIFFERENTIATION AND FUNCTION OF IMMUNOLOGIC DEFENSES

An understanding of the relation of immunologic defects and associated superinfections to lymphoreticular diseases requires an appreciation of the normal process of differentiation and maturation of host immunologic defenses. Based on clinical observations (2–6) and extensive studies in experimental animals (7–12), the following series of events seems likely. In late fetal life lymphoid stem cells are differentiated in one of two "central lymphoid organs" (13, 14). Some are processed in the thymus and others in the mammalian counterpart of the bursa of Fabricius in the chicken, probably the Peyers patches in the lower gastrointestinal tract (15, 16).

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[1] "Superinfection" is used here to connote infection occurring in patients with an underlying disease process.

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The differentiating action of the thymus appears to be mediated by a humoral substance elaborated by epithelial elements (17). The lymphoid cells differentiated in the thymus migrate to peripheral lymphoid areas to populate the subcortical regions of lymph nodes and the white pulp of the spleen (18, 19). These cells, which constitute the cellular or "thymic-dependent" immune system, possess a recognition mechanism (20) and can mediate cellular immune functions such as delayed hypersensitivity and graft rejection (21).

The second population of lymphocytes, differentiated at a site other than the thymus, also have a recognition mechanism but migrate to populate the germinal centers and medullary cords of the lymph nodes, the red pulp and germinal centers of the spleen, the bone marrow, and the intestinal mucosa (18, 19). These cells constitute the humoral or "thymic-independent" immune system. Both as lymphocytes and by transformation to plasma cells they mediate the production of humoral antibodies (e.g., opsonins, bacteriolysins) (9, 21). The cells of both systems are in constant circulation (18, 22) but the cells of the thymic-dependent or cellular-immune system seem to have a longer life span and to constitute a majority of the circulating lymphocytes (22, 23). Although thymic-dependent lymphocytes do not make circulating antibody, recent evidence suggests that they may play a "helper" role in stimulating antibody production by thymic-independent lymphocytes (24, 25).

LYMPHORETICULAR DISEASES ASSOCIATED WITH IMMUNOLOGIC DEFICIENCY

It is clinically useful to divide the lymphoreticular diseases into those in which the tissues involved mediate humoral, cellular, or both types of immunologic responses. Lymphoreticular diseases with defects in humoral immunity are generally associated with superinfection by a different spectrum of organisms than are those with defects in cellular immunity (7). This fact has important implications for initial therapy, choice of diagnostic procedures, and prognosis in patients with lymphoreticular disease suspected of having a superinfection. The following classification of diseases by their type of immunologic deficit is made with the recognition that some overlap in deficits occurs. Also, in these and other chronic progressive diseases there may be deficiency of both humoral and cellular immune defenses as wasting and iatrogenic intervention increase (26, 27).

Diseases with defects in humoral immunity.—Deficits in humoral immunity occur in pure form in sex-linked recessive congenital agammaglobulinemia first described by Bruton (28–30). As might be predicted from the pathologic observation of the absence of plasma cells in the lymph nodes, bone marrow, and intestinal mucosa in these patients, there is production of only trace amounts of immunoglobulins. A similar defect occurs in "acquired" agammaglobulinemia which has its onset in later childhood or adult life. The term "acquired" may be misleading since an increasing body of evidence suggests that despite its late onset, the disease is genetically determined in many patients (10, 31–33). In addition to their defect in humoral immunity some patients with "acquired" agammaglobulinemia have an additional deficiency in cellular immunity (31, 32, 34). Strangely, defects in humoral immunity have been demonstrated in some patients with thymomas (35). Although apparently mild defects in cellular immunity have also been found in some of these patients (35), the paradox of failure of the thymic-independent system in thymic neoplasm is unexplained. In a heterologous group of syndromes, best termed the "dysgammaglobulinemias," there are deficiencies or excesses, or both, of some of the immunoglobulin classes (36–39). Lymphoproliferative disorders, especially macroglobulinemia and multiple myeloma, are frequently associated with an increased incidence of infection and either qualitative or quantitative defects in immunoglobulin production (37, 40, 41). The immunoglobulin produced, often in great excess, may have specificity for certain bacterial antigens (e.g., pneumococcal polysaccharide, streptolysin, 42). Nevertheless, patients with multiple myeloma or other diseases with humoral immune deficits are usually unable to sustain adequate levels of protective antibodies or to mount an effective humoral immunologic offensive when invaded by pathogenic microorganisms.

Diseases with defects in cellular immunity.—The importance of the thymus in the development of a competent cellular immune system, as suggested by experiments in animals, has been confirmed by recent studies of immune function in patients with failure of development of the anlage of the thymus, the embryonic pharyngeal pouches. In these patients with "pharyngeal pouch" syndromes, dysgenesis of the thymus has occurred alone (2, 43, 44) or in association with failure of development of the thyroid (45) or parathyroid glands (DiGeorge's syndrome, 46, 47). These syndromes have in common thymic dysgenesis with a paucity of lymphocytes in the peripheral blood, lymph nodes, and intestinal mucosa; failure of cellular, but not humoral immune defenses; and frequent infection with those viruses, bacteria, and fungi normally antagonized by the cellular immune system (43, 46). Another syndrome with failure of cellular immunity but with normal serum immunoglobulins is the "cartilage-hair hypoplasia" syndrome. Children with this disease have short-limbed dwarfism, abnormally fine hair, abnormal tests of cellular immune function, and an apparent predisposition to fatal or severe varicella (48, 49). Although these diseases are most instructive, they present as clinical problems less frequently than two other diseases with deficits of cellular immunity, sarcoidosis and Hodgkin's disease. The defect in cellular immunity (and presumably the increased susceptibility to infection) may precede the onset of clinical signs of sarcoidosis and persist after their disappearance (50, 51). In individual cases the distinction between sarcoidosis with superinfection and primary mycobacterial or fungal infection may be most difficult (52). In Hodgkin's disease, too, the development of a defect in cellular immune function may be present in the absence of symptoms of the disease and is associated with a poorer prognosis (41, 51, 53, 54). The defect in cellular immunity in such patients is usually recognized when it is found that they have lost tuberculin sensitivity or fail to develop delayed hypersensitivity to dinitrofluorobenzene (55). Equally important clues to a defect in cellular immunity in such patients are the frequency with which they have infections and the spectrum of infecting agents.

Diseases with defects in both humoral and cellular immunity.—Deficiencies in both humoral and cellular immunity are represented in their purest form in "Swiss type" autosomal recessive lymphopenic agammaglobulinemia (3, 56). Patients with this disease have absent or aplastic thymic tissue, lymphopenia, and marked depletion of lymphocytes in all lymphoid tissues. Thus, they are unable to respond to antigenic stimuli by production of circulating antibody and fail to develop delayed allergy or to reject tissue homografts. Prior to the successful immunologic reconstitution in such patients (to be discussed later) they usually died in the first year of life from multiple infections of the skin, bronchopulmonary tract, or gastrointestinal tract (3). Another form of lymphopenic agammaglobulinemia appears to differ from the "Swiss type" in having sex-linked recessive inheritance, less severe lymphoid depletion, and slightly longer life expectancy (38, 56, 57). In a still milder condition, lymphopenic thymic dysplasia with dysgammaglobulinemia, there is a deficiency of cellular immunity but some of the immunoglobulin classes may be present in normal concentrations (56, 58). The Wiskott–Aldrich syndrome is characterized by thrombocytopenia, eczema, defects in both cellular and humoral immunity, and frequent infections (59, 60). Although patients with this disease may have normal levels of immunoglobulins, they are unable to respond to certain polysaccharide and lipopolysaccharide antigens, perhaps due to a defect in the processing or recognition of antigen (59–61). Another disease with seemingly unrelated components is ataxia telangiectasia. The clinical manifestations include cerebellar ataxia, oculocutaneous telangiectasia, and frequent infections of the sinuses and respiratory tract. There is lymphoid depletion of the thymus, lymphopenia, and usually a deficiency of IgA and IgE (62, 63).

The most frequent clinical setting for superinfection in lymphoreticular diseases with defects in both cellular and humoral immunity is in patients with chronic lymphatic leukemia, lymphosarcoma, or reticulum cell sarcoma. Many patients with chronic lymphatic leukemia have attenuated primary and secondary antibody responses, some loss of previous delayed hypersensitivity, and resistance to sensitization with agents normally stimulating delayed hypersensitivity (41). The defects in lymphosarcoma and reticulum cell sarcoma are variable but generally tend to be milder than those in chronic lymphatic leukemia (41).

Decreases in host cellular and humoral immunity with increased susceptibility to superinfection may be found not only in association with "naturally occurring" lymphoreticular diseases but also in association with iatrogenic immunologic disease induced by steroids (64), antilymphocytic or antithymic serum (65–67), or other immunologic suppressive agents (27). Similarly, many of the physical or chemical means of treating some lymphoreticular diseases may diminish cellular and humoral host defenses and predispose to superinfection (27).

INFECTING MICROORGANISMS ASSOCIATED WITH LYMPHORETICULAR DISEASES

The predisposition to infection of patients with lymphoreticular disorders seems to follow from their demonstrated defects in host immune mechanisms, be they cellular or humoral. The specialized role these systems play in host defense is suggested by the difference in the spectrum of infecting agents occurring when one or the other system is deficient. Therefore, knowledge of the type of immunologic deficit enables the physician to predict more accurately the type of infection his patient might have (Table 1).

Diseases with defects in humoral immunity.—The prototype for this group of illnesses is the Bruton-type congenital agammaglobulinemia. Virtually devoid of circulating antibody, such patients are frequently infected by common pyogenic microorganisms. Dr. Bruton's original patient had documented pneumococcal sepsis nine times during a four-year period (28, 29). At any time after age eight months, when stores of maternal antibody are depleted, the characteristic syndrome of recurrent severe infections may ensue. Most frequently, pneumococci, coagulase-positive staphylococci, meningococci, *H. influenzae,* and sometimes group A streptococci and salmonella are the responsible bacterial pathogens. Because these patients cannot muster any antibody response, the same microorganism may be responsible for serial infections. For example, recurrent infections with the same capsular type of pneumococcus often have been noted. This is due to the failure of development of specific anticapsular antibodies necessary for rapid pneumococcal phagocytosis. Pneumonia, otitis media, pyoderma, purulent conjunctivitis, meningitis, septic arthritis, and pharyngitis are seen frequently and may present with unusually high fever and marked leukocytosis (7). These patients combat most viral infections effectively but progressive vaccinia (68) and severe varicella have occasionally been noted. The vaccine

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TABLE 1. CORRELATION OF THE LYMPHORETICULAR DISEASES, THEIR IMMUNOLOGIC DEFICITS, AND THEIR CHARACTERISTIC SUPERINFECTING MICROORGANISMS

Diseases	Immunologic defect	Superinfecting organisms
Bruton's agammaglobulinemia "Acquired" agammaglobulinemia Dysgammaglobulinemias Macroglobulinemia Multiple myeloma	Failure of humoral immunity (insufficient production of circulating antibodies)	*Diplococcus pneumoniae* *Staphylococcus aureus* *Neisseria meningitidis* *Hemophilus influenzae* Varicella virus Vaccinia virus *Pneumocystis carinii* *Giardia lamblia*
"Pharyngeal-pouch" syndromes including DiGeorge's syndrome Cartilage—hair hypoplasia syndrome Sarcoidosis Hodgkin's disease	Failure of cellular immunity (loss of delayed hypersensitivity and graft rejection)	*Mycobacterium tuberculosis* *Cryptococcus neoformans* *Histoplasma capsulatum* *Nocardia asteroides* Cytomegalovirus Varicella virus Vaccinia virus *Toxoplasma gondii*
"Swiss type" lymphopenic agammaglobulinemia Sex-linked recessive lymphopenic agammaglobulinemia Lymphopenic thymic dysplasia with dysgammaglobulinemia Wiskott–Aldrich syndrome Ataxia telangiectasia Chronic lymphatic leukemia Lymphosarcoma Reticulum-cell sarcoma	Variable defects in both humoral immunity and cellular immunity	Infections characteristic of both of the above defects are seen

strain of Type 1 poliomyelitis virus is reported to have produced paralytic illness in a child with agammaglobulinemia (69). The protozoan, *Pneumocystis carinii,* has produced a progressive interstitial pneumonia in such patients, resulting in striking impairment of arterial oxygen saturation (70). Although patients with the "acquired" form of agammaglobulinemia generally have somewhat higher titers of immunoglobulins, they also suffer recurrent infections, particularly of the paranasal sinuses and lungs, leading to bronchiectasis and recurrent life-threatening pneumonia (31, 33). Bacterial infections may also present as hepatic abscesses and pyoderma in these patients (31). In two recent studies, the authors found the intestinal flagellate, *Giardia lamblia,* in the stools of approximately half of their study population with "acquired" immunoglobulin deficiency (31, 71). This rate, vastly greater than that seen in normal individuals in the United States, has not been explained.

The defective antibody production in patients with monoclonal gammopathies and multiple myeloma seems to make them particularly susceptible to pneumococcal pneumonia (37, 41, 72). In fact, the diagnosis of the underlying disorder may first be made when an alert physician obtains a serum protein electrophoresis on a patient with "pneumonia and anemia." In addition, patients with myeloma are susceptible to bacterial infections of the skin and genitourinary tract (41, 72).

Diseases with defects in cellular immunity.—Whereas patients with defects in humoral immunity develop infections primarily with pyogenic "extracellular" microorganisms, patients with defects in cellular immunity suffer infections from microorganisms thought of as "intracellular" parasites: the tubercle bacillus, fungi, and viruses.

In its pure form this immunologic defect is exemplified by the various thymic dysgenesis or "pharyngeal pouch" syndromes such as DiGeorge's syndrome (46). These children are conspicuous in their inability to contain viral infections which usually are controlled easily by normal persons. It has been suggested that the first clinical expression of the underlying disease may be the patient's inability to cope with a viral infection (73). Overwhelming measles, virus pneumonia (74), and progressive vaccinia (68, 75), among others, have been documented.

Defects in cellular immunity are most frequently observed in Hodgkin's disease (41, 76). It is said that "tuberculosis follows Hodgkin's disease like a shadow" (77). Indeed, the association is so frequent that for years the tubercle bacillus was thought by many to be the etiologic agent in Hodgkin's disease (77). In accord with the experience of others (53, 76), we have noted several patients with Hodgkin's disease who subsequently developed histoplasmosis, cryptococcosis, nocardiosis, or candidiasis. It has been recently reported that disseminated sporotrichosis also may occur in such a setting (78). Generalized infections with members of the herpes virus group, especially varicella virus, and cytomegalovirus have been recognized frequently (53, 79, 80). Sepsis is a common event during the terminal course of the illness (79). In addition, several reports have indicated that patients with Hodgkin's disease may harbor multiple severe infections simultaneously: toxoplasma encephalitis and herpes simplex pneumonia (81) and herpetic esophagitis, cytomegalovirus pulmonary infection, toxoplasmosis of the lung and stomach, and sarcocystis involvement of the myocardium (82).

Diseases with defects in both humoral and cellular immunity.—Those diseases with defects in both humoral and cellular immunity occurring primarily in adults include chronic lymphocytic leukemia, lymphosarcoma, and reticulum cell sarcoma (41, 79, 83, 84). Patients with these diseases acquire infections characteristic of both types of defects. Pneumococcal pneumonia and staphylococcal furunculosis occur frequently (79, 84). Pyelonephritis and septicemia with Gram-negative bacteria, especially *Proteus* and *Klebsiella-Enterobacter* species, are also a problem (79, 83). In addition, these patients develop opportunistic fungal infections with *Candida, Aspergillus,* or *Mucor* and may suffer infection due to the cytomegalovirus, herpes simplex or vaccinia virus (79, 85).

CLINICAL FEATURES OF INFECTION COMPLICATING LYMPHORETICULAR DISEASES

Considering the wide spectrum of infectious illness already mentioned, it is difficult to derive general clinical principles that will be apropos to all situations. Several features deserve emphasis, however.

A severe infection resulting from immunization may be the physician's first clue to an underlying immunologic disorder. Smallpox vaccinations that progress to vaccinia gangrenosa, BCG immunizations that disseminate, and polio immunizations that produce paralysis are such examples (68, 69, 75, 86). With the widespread use of live measles and rubella vaccines it is possible that disseminated disease caused by these viruses also may be seen in patients with immunologic defects. Further, the questionable practice of using smallpox vaccination to terminate herpes simplex infection should be discontinued. All too frequently these patients have their herpes simplex infection as a result of an underlying lymphoreticular malignancy with an immune defect and die of the unchecked spread of vaccinia virus from the vaccination site (68, 85).

A vexing feature of infections in lymphoproliferative disease is the frequent difficulty in recognizing their presence. Especially in adults, life-threatening infections occur more often late in the course of the underlying disease. At this stage it is difficult to separate the manifestations of the infection from those of the primary disease and the effects of therapy. It is not surprising then, that many such infections are revealed only at autopsy (79). Therefore a compulsive search for infection should be undertaken whenever a patient's course takes a downward turn.

TREATMENT OF SUPERINFECTIONS IN LYMPHORETICULAR DISEASES

Successful treatment of infection in individuals with lymphoreticular disorders ideally requires (*a*) precise identification of the infecting microorganism; (*b*) proper utilization and choice of antimicrobial agents; and (*c*) correction or alleviation of the underlying immunologic defects. While these goals should always be sought, they will in many instances be unattainable.

Diagnosis.—Precise diagnosis of the etiologic agent(s) responsible for infection in a patient with a lymphoreticular disease often presents a difficult problem. Frequently one is faced with a febrile patient without localizing signs of infection; or a patient with a local or diffuse pneumonic infiltrate who is bringing up little or no sputum, or in whom the sputum yields no pathogenic microorganism; or an individual in whom the cerebrospinal fluid shows an abnormal number of cells but no identifiable microorganism on smear, india ink preparation, or culture. These clinical circumstances are often discouraging, yet a recent review of 67 febrile episodes in 37 patients with chronic lymphocytic leukemia at the Vanderbilt University Hospital indicated that an infectious agent could be presumptively identified as the cause of fever in 44.8 percent of these episodes (87). Significantly, in the 37 episodes of fever for which no cause could be determined, adequate diagnostic studies had been obtained in only 5. It is thus clear that only careful utilization of available diagnostic techniques will provide the physician with an optimal opportunity to identify precisely infecting microorganisms in patients with lymphoreticular disorders.

Specimens of blood, bone marrow, sputum, urine, and spinal fluid for routine microbiologic examination must be obtained with great care. These examinations should include the cultivation of blood in thioglycollate broth (which will support the growth of anaerobes) and sputum on appropriate media in an anaerobe jar (88, 89). Careful cultures for fungi and acid-fast bacilli should also be obtained. We have found urine cultures after prostatic massage useful in the diagnosis of cryptococcosis, blastomycosis, and histoplasmosis. The recovery of *Histoplasma capsulatum* from the sputum is greatly facilitated by injecting a sample into mice, which are then sacrificed after three weeks and their livers and spleens cultured. *Nocardia* species are readily grown on blood agar plates, but these must be retained for at least ten days. These microorganisms also grow well on media used to culture fungi and, contrary to previous beliefs, appear to survive the digestive procedures utilized in mycobacterial cultures (90, 91).

Peripheral blood, sputum, urine, and marrow may be Gram-stained to determine the presence of bacteria and some fungi. The Wright's stain is particularly helpful in recognizing *H. capsulatum* in macrophages, and we have found the Gram stain and incomplete acid-fast stain most useful in indicating the possibility of nocardial infection (91). India ink preparations should be performed on the sediment from centrifuged spinal fluid in an attempt to diagnose cryptococcal meningitis.

When viral diagnostic procedures are available, consultation with the virologist will facilitate obtaining the proper specimens for identification of infection with cytomegalovirus, herpes simplex, varicella virus, vaccinia virus, and other viral agents.

Where roentgenograms reveal the presence of pleural effusion or pneumonic infiltrates, or both, pleural biopsy (92, 93), needle biopsy of the lung

(94, 95), needle aspiration of the lung (96, 97), and even open-lung biopsy (98) may be helpful in establishing the cause of infection if the patient's general condition and hematologic status permit these procedures.

Few serological procedures are useful in providing immediate diagnostic information as the antibody levels in at least two specimens are generally required for adequate interpretation. In addition, patients with defects of their humoral immune systems may be unable to respond to antigenic stimuli. Nevertheless, elevated titers of complement-fixing antibodies to the yeast and mycelial antigens of histoplasma, blastomyces, and coccidioides may be used as presumptive evidence of infections with these fungi (99, 100), though care must be taken in interpretation of these tests if previous skin tests have been performed (101, 102). Tests for the presence of cryptococcal antigen appear to be useful in making the diagnosis of active cryptococcal infection even when india ink preparations or spinal fluid cultures are negative (103, 104). The Sabin–Feldman dye test and the toxoplasma complement-fixation test may provide useful information in the diagnosis of acquired toxoplasmosis (105, 106).

Specific antimicrobial therapy.—Febrile and toxic patients with lymphoreticular disorders must often be treated with multiple antimicrobial agents unless immediate diagnostic measures precisely identify the infecting agent. Thus, for presumed bacterial infection, initial regimens often include one of the penicillinase-resistant penicillins, cephalosporins or ampicillin, plus agents effective against Gram-negative bacilli such as kanamycin, colistin, polymixin B, or gentamicin. Carbenicillin may have promise in treating pseudomonas infections, especially when combined with gentamicin, though resistant strains have occurred (107–109). The rôle of polyvalent pseudomonas vaccines in the prevention or treatment of pseudomonas infections must be further evaluated, though initial studies in burn patients appear promising (110).

Amphotericin B is the drug of choice for treating histoplasmosis, cryptococcosis, candidiasis, and mucormycosis. Because of the toxicity of this agent, most clinicians do not use it in therapy unless a presumptive diagnosis of one of these infections has been made. There are, however, several therapeutic approaches that minimize its toxicity (111, 112). Treatment with amphotericin B and iodides has been disappointing in disseminated aspergillosis, but local application via bronchial lavage, aerosol, or small catheters passed into cavitary lesions has on occasion been successful (113–115).

A new oral antifungal agent, 5-fluorocytosine, has shown promise in treating candidiasis and cryptococcosis in animals and in a few instances, in man (116, 117). Sulfonamides given so that the serum sulfonamide levels are maintained between 12 and 15 mg percent are the treatment of choice for nocardiosis (91).

The chemotherapeutic approach to the therapy of viral infections is in its infancy. In patients with markedly altered host resistance, the thiosemicarbazones have been helpful in treating disseminated vaccinia infections (118, 119), and there are a few reports concerning the use of cytosine arabinoside in treating severe herpes zoster (120). As previously mentioned, smallpox vaccinations should never be utilized to treat herpes simplex infections (fever blisters), especially in individuals with defective immune systems (68, 85).

Pyrimethamine and sulfonamides have been advocated for the treatment of acquired toxoplasmosis (106). Similar therapy has been used in *Pneumocystis carinii* infection (121), but most investigators feel that pentamidine isethionate is the drug of choice (95, 122–124).

Therapy of underlying disorder.—It is clear that any treatment which can favorably affect the underlying lymphoreticular disorder will aid in the prevention or therapy of complicating microbial infections. Thus, X-ray treatment and the use of cytotoxic drugs may on occasion produce remissions or even "cures" of malignant diseases of the lymphoreticular organs. However, such modalities of therapy must always be viewed as two-edged swords because, as outlined above, they may also reduce the host's ability to combat infection.

Replacement therapy with pooled gamma globulin may be useful in reducing the number of infections in patients with defective humoral defense mechanisms (125). However, a more recent and exciting approach to the therapy of some disorders of host immunity consists of actual immunologic reconstitution. Infants with DiGeorge's syndrome (and thus defective cellular immunity due to aplasia of the thymus) have had immunological competence restored by implantation of fetal thymic tissue (126, 127). Even more impressive has been the immunologic reconstitution of defects of both cellular and humoral immunity in patients with sex-linked and autosomal recessive (Swiss type) lymphopenic agammaglobulinemia (128–130) and in a patient with the Wiskott–Aldrich syndrome (131). Such reconstitution, without a lethal graft-versus-host reaction, was accomplished with blood leukocytes and marrow cells only by careful histocompatibility matching.

Finally, it is intriguing to speculate how Lawrence's fascinating studies with transfer factor (132) might be used in treating patients with deficient cellular immunity. Lawrence and his colleagues have conferred delayed hypersensitivity to tuberculin in tuberculin-negative subjects by the injection of leukocytes or even cell-free extracts of leukocytes from tuberculin-positive individuals. This technique has been used to transfer tuberculin sensitivity to tuberculin-negative patients with malignant lymphomas (133) and has been adapted by Kempe in successfully treating a child with disseminated vaccinia (132). It will be interesting to watch for further developments in this mode of therapy.

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INTRALEUKOCYTIC MICROBICIDAL DEFECTS[1,2] 7003

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In recent years, a new methodology has evolved which attempts to dissect the various parameters of leukocyte[3] function in patients with an increased susceptibility to infection. The result has been the elucidation of a number of clinical conditions in which the leukocyte response is inadequate. Consideration will be given here to those conditions in which a defect in the intraleukocytic microbicidal mechanisms results in the prolonged intracellular survival of ingested organisms. The organisms are protected from the microbicidal systems of serum and from the lethal effect of certain antibiotics as a consequence of their intracellular residence (1–4); multiplication and dissemination occur with a prolongation and accentuation of disease. In general, phagocytosis is normal in these conditions as is the ability of the leukocytes to respond in normally adequate numbers with directed movement to the site of microbial invasion. Neutrophil dysfunction syndromes associated with defective migration or phagocytosis have been described (for reviews see 5, 6); they will not be considered here.

THE PHAGOCYTIC PROCESS

Phagocytosis by granulocytes initiates a sequence of morphological and biochemical events which lead, in most instances, to the death of the ingested organisms. The process begins with the incorporation of the organism into a phagocytic vacuole lined by the invaginated cell membrane. The phagocytic vacuole and adjacent granules fuse; the connecting membranes rupture and the contents of the granules pass into the internalized extracellular space of the phagocytic vacuole (7, 8).

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[2] The following abbreviations are used: CGD (chronic granulomatous disease); GSH (reduced glutathione); GSSG (oxidized glutathione); HMP (hexose monophosphate pathway); MPO (myeloperoxidase); NAD^+ (nicotine adenine dinucleotide); NADH (reduced nicotine adenine dinucleotide); $NADP^+$ (nicotine adenine dinucleotide phosphate); NADPH (reduced nicotine adenine dinucleotide phosphate).

[3] This review will deal only with the neutrophil and with the corresponding cell in certain laboratory animals, the heterophil.

The leukocyte granules were identified as lysosomes by their content of the acid hydrolytic enzymes generally found in these organelles (9). They contained in addition, enzymes not characteristic of lysosomes, e.g., alkaline phosphatase (9), MPO, (10, 11), and neutral or alkaline proteases (12). This discrepancy is explained in part by the presence of two or more populations of leukocyte granules which differ in size, density, time and mode of origin and enzymic content. This heterogeneity is apparent on morphological and histochemical examination of intact cells and by separation of isolated granules into distinct fractions.

The first granules seen in the developing rabbit heterophil originate from the proximal or concave face of the Golgi complex (13). They are relatively large (ca 800 mμ), dense and azurophilic (13, 14) and contain acid phosphatase as well as other acid hydrolytic enzymes (15–19), and thus are primary lysosomes. They also contain sulfated mucopolysaccharide (20, 21), large deposits of pyroantimonate precipitable cation, presumably sodium (22), basic protein (20, 21), MPO (16–19, 23), and one-third of the lysozyme (18, 19). They have been named the azurophil (13), primary (14), or A (18) granule. Although the sole granular type in the progranulocyte, they make up only 10–20 percent of the granules of the mature cell (13). As the leukocyte develops, a smaller (ca 500 mμ) and less dense granule appears (13, 14). This specific (13), secondary (14), or B (18) granule is derived from the distal or convex face of the Golgi complex (13). It is devoid of acid hydrolytic enzymes and thus is not a lysosome. It contains alkaline phosphatase (15–19), lactoferrin (24), two-thirds of the lysozyme (18, 19), and relatively light deposits of pyroantimonate precipitable cation (22). It is the predominant granule of the mature cell. A third granular type, the tertiary granule has been seen in electron micrographs of mature leukocytes (14). These small (ca 300 mμ) pleomorphic granules contain acid mucosubstance and acid phosphatase (8, 15), and may correspond to the C particles isolated by Baggiolini, Hirsch & de Duve (18, 19). Zeya & Spitznagel (25) also have separated leukocyte granules into three fractions. Fraction III was intensely eosinophilic owing to its content of cationic proteins but, unlike C particles, contained no acid phosphatase. Baggiolini, Hirsch & de Duve (19) described a fourth (D) fraction made up of large empty vesicular membrane structures and containing 10 percent of the alkaline phosphatase and all of the acid *p*-nitrophenyl phosphatase, an enzyme which differs from the lysosomal acid phosphatase. Although rabbit heterophil granules have been studied most extensively, other species including man (8, 21–23, 26), cat (27), rat (28), guinea pig (29), horse (30), chicken and pigeon (31) also show granular heterogeneity. Species differences in the structure and content of leukocyte granules, however, exist.

It is probable that all or most of the granules can participate in the degranulation process since extensive phagocytosis results in the virtual disappearance of cytoplasmic granules. It is not clear whether degranulation occurs randomly or sequentially, with the rupture of one type of granule preceding that of another. The mechanisms involved in granule fusion and lysis are unknown. Leukocytes during phagocytosis have a marked tendency to clump which may be due to a chemical alteration of the cell surface or to the interdigitation of projections from adjacent leukocytes (32), or perhaps both. The membrane of the phagocytic vacuole is invaginated cell membrane and thus may acquire the same adhesive qualities of the surface membrane. Lysis may be facilitated by the fall in intravacuolar pH (see below) since isolated granules rapidly lyse at pH levels below 5.5 (9). Leukocyte granules also are lysed by lysolecithin (33), a substance which may be formed by granular phosphodiesterase (34) or be absorbed from the extracellular fluid (35). Artificial phospholipid membranes (liposomes) have permeability properties similar to those of natural membranes (36). Studies of the interaction of leukocyte granules with liposomes have led to the postulate that unmasked negatively charged phospholipid groups of the phagocytic vacuole membrane react with proteins of the granular membrane leading to membrane fusion and rupture (37). Lipid peroxidation of the connecting membranes has been suggested (38).

THE METABOLIC BURST

The phagocytic process is associated with a burst of metabolic activity (39, 40). Oxygen consumption is considerably increased (41–43). Respiration in the absence of exogenous substrate is due, in part at least, to glycogen breakdown since glycogen levels fall during phagocytosis (41–43). Glucose consumption and lactic acid production also are increased (41–43), particularly under anaerobic conditions. The phagocytosis-induced increase in glucose 1-C oxidation, a measure of the oxidation of glucose via the HMP, is particularly striking (42, 44–47). The respiratory burst occurs very early following contact between particle and cell preceding extensive phagocytosis and degranulation (48, 49). Early swelling and intracytoplasmic lysis of "medium dense" granules have been reported (48, 49). The respiratory burst is associated with H_2O_2 formation (45, 50–52) and with the reduction of nitroblue tetrazolium (NBT) to blue formazan (53).

Lipid turnover is increased by phagocytosis (54, 55). The increased incorporation of orthophosphate into phospholipids is limited to minor acidic phosphatides (56, 57). The increased conversion of lysolecithin to lecithin and of lysophosphatidylethanolamine to phosphatidylethanolamine during phagocytosis suggests that plasma lysocompounds may be the precursors for the major leukocyte phospholipids (35). Cellular triglyceride acts as the fatty acid donor for the acylation reaction (58). Phagocytosis has no effect on protein synthesis (55); RNA turnover, however, is increased (59). The metabolic burst is independent of RNA synthesis (60).

The enzymic mechanisms responsible for the metabolic burst have been the subject of considerable study and not inconsiderable controversy. A satisfactory scheme must account for the important triad of increased O_2 consumption, increased H_2O_2 production, and increased HMP activity. Two en-

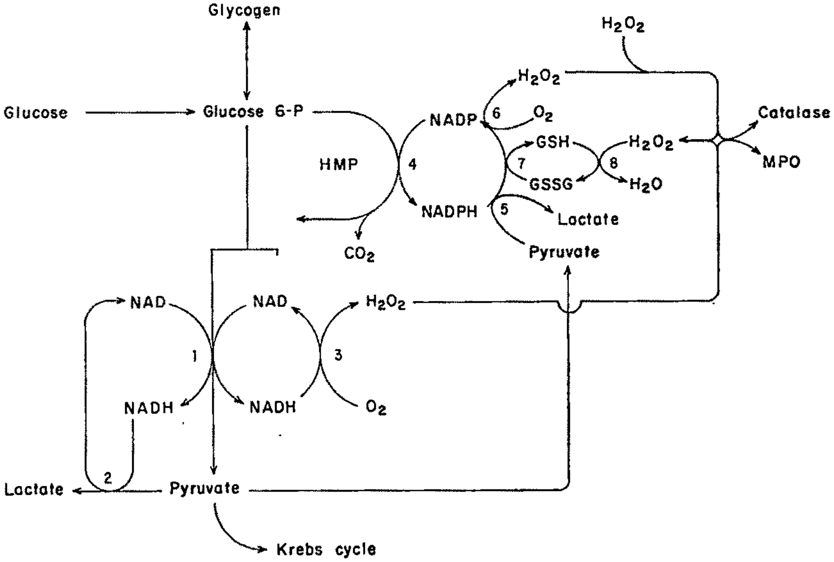

FIGURE 1. Leukocyte metabolism. 1—phosphoglyceraldehyde dehydrogenase; 2—NADH-linked lactic dehydrogenase; 3—NADH oxidase; 4—glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; 5—NADPH-linked lactic dehydrogenase; 6—NADPH oxidase; 7—GSSG reductase; 8—GSH peroxidase.

zymes of the HMP, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase reduce $NADP^+$ to NADPH and the continued activity of this pathway is dependent upon the reoxidation of NADPH. Thus, interest has centered on two aspects of the enzymic mechanism for the respiratory burst; 1. the primary oxidase which accounts for the increased O_2 consumption and H_2O_2 production, and 2. the link between this oxidase activity and the increased NADPH oxidation required for the continued operation of the HMP (Fig. 1).

PRIMARY OXIDASE

The early metabolic changes in the phagocytizing granulocyte are associated with the oxidation of the reduced pyridine nucleotides (61). The $NADP^+$/NADPH ratio is significantly increased by phagocytosis, whereas the NAD^+/NADH ratio is unaffected (61, 62), suggesting that the oxidation of reduced pyridine nucleotides in intact cells reflects NADPH rather than NADH oxidation. These data are also compatible with a rapid reduction and re-oxidation of NAD^+ tightly coupled to NADPH oxidation. Oxygen consumption by resting leukocytes is inhibited approximately 70 percent by 1 m*M* cyanide (42, 43, 63). Granulocytes contain a few mitochondria and the cyanide-sensitive respiration in resting cells may reflect the activity of the cytochrome system in these organelles. The phagocytosis-induced respiratory burst, however, is largely unaffected by 1 mM cyanide (41, 42, 63). Thus, interest has centered on a cyanide-insensitive NADPH or NADH oxidase as the primary oxidase.

NADPH oxidase.—Iyer & Quastel (64) found an enzyme in guinea pig leukocyte homogenates which catalyzed the oxidation of NADPH and, to a lesser degree, NADH by O_2 with the formation of H_2O_2. This enzyme was strongly stimulated by Mn^{++} and had a pH optimum of 5.5. It was localized by Rossi & Zatti (63) in the granular fraction of the cell. Specific activity was increased by phagocytosis (63, 65); however, the increase in specific activity (units per milligram protein) (63) may result in part from a decrease in cell protein rather than an increase in total NADPH oxidase activity (40, 66).

Peroxidases, including MPO (67, 68), can catalyze the oxidation of NADH and NADPH by O_2 with the formation of H_2O_2 (69). This oxidative reaction is strongly stimulated by Mn^{++} and by certain phenolic compounds (69), e.g., thyroid hormones (67) and estrogens (67, 70). The H_2O_2 formed can be employed as the oxidant in coupled oxidations (68). Roberts & Quastel (71) suggested that the enzyme in leukocyte homogenates which catalyzed the aerobic oxidation of NADH and NADPH was peroxidase. Under their assay conditions, enzyme activity was inhibited 80 percent by 1 mM cyanide, a known peroxidase inhibitor. The reduction of NAD^+ by the lactic dehydrogenase of leukocyte granules and its reoxidation by MPO has been demonstrated (72). The identification of MPO as the primary oxidase however, was brought into question by the resistance of the metabolic burst to inhibition by 1 mM cyanide (41, 42, 63), and by the presence of a respiratory burst in the leukocytes of a patient with a genetic absence of MPO (73).

The NADPH oxidase activity of leukocyte granules although depressed is not completely inhibited by 1 mM cyanide (74). Activity in the presence of cyanide is associated with H_2O_2 formation (75). The cyanide-insensitive (presumably nonperoxidase) granular NADPH oxidase was therefore suggested as the primary oxidase (74). Other investigators consider its activity inadequate to account for the respiratory burst (66). The nature of this enzyme has not yet been established by isolation, purification, and characterization. MPO may not be completely inactivated by 1 mM cyanide, particularly at the high concentrations present in the cell (71, 76).

NADH oxidase.—An alternative enzyme, NADH oxidase, has been proposed as the primary oxidase (77–79). It is a flavoprotein which reduces O_2 to H_2O_2. It is soluble when extracted with isotonic alkaline KCl but is 50 percent granular and 50 percent soluble when 0.34 M sucrose is employed (66). The localization of the enzyme in the intact cell is uncertain. The enzyme is insensitive to 1 mM cyanide and its pH optimum is 4.5–5.0. It has a high K_m for NADH, suggesting that the rate of the reaction will be dependent upon the NADH concentration over a wide range. Activity is greater in guinea pig than in human cells as is the respiratory burst, and is increased by phagocytosis in both species (66). It is present also in rat leukocytes (80). Both the NADH oxidase activity of extracts and the respiration of intact leukocytes are inhibited by quinacrine (79) and hydrocortisone sodium succinate (81). Enzyme activity was considered adequate to account for the respiratory burst (66).

D-Amino acid oxidase.—D-Amino acid oxidase is present in the granular fraction of human and guinea pig leukocytes (82). This cyanide-insensitive enzyme catalyzes the oxidation of D-amino acids by O_2 with the formation of H_2O_2. D-Amino acids are foreign to mammalian tissues; however, they may be provided by ingested microorganisms (82). The respiratory burst associated with particles which do not contain D-amino acids, e.g., latex, cannot be explained in this way (82).

LINK TO NADPH OXIDATION

The oxidation of NADPH by the primary oxidase would meet the total requirements of the respiratory burst, i.e., increased oxygen consumption, H_2O_2 formation, and NADPH oxidation. A primary oxidase other than NADPH oxidase, however, requires a link to NADPH oxidation. An NAD^+–NADPH transhydrogenase reaction is one possibility. A transhydrogenase has been detected in human leukocytes (83) but is minimal in guinea pig cells (66). Dehydroascorbate–NADPH reductase also was detected in human leukocyte granules; however, its rate was considered inadequate to account for the increase in HMP activity (66).

NADPH-linked lactic dehydrogenase.—NADH generated by glycolysis is generally reoxidized by lactic dehydrogenase. It has been suggested (77) that in guinea pig leukocytes activation of NADH oxidase by phagocytosis provides an alternate means for NADH oxidation, and that under these conditions NADPH is oxidized by an NADPH-linked lactic dehydrogenase. This enzyme has been found in guinea pig (77) but not in rat (80) or human (66) leukocytes. Its role in guinea pig cells has been questioned (47, 63, 84, 85). An NADH-linked lactic dehydrogenase has been found in or on leukocyte granules (86).

The glutathione system.—The importance of H_2O_2 in the stimulation of glucose 1-C oxidation has recently been emphasized. Cyanide, azide, and aminotriazole, which inhibit MPO and catalase and thus the utilization or destruction of H_2O_2, stimulate glucose 1-C oxidation by phagocytizing rat leukocytes (80, 87). That this is owing to the accumulation of H_2O_2 is suggested by the stimulation of glucose 1-C oxidation by added H_2O_2 (87). Stimulation by H_2O_2 is prevented by the sulfhydryl blocking agent, N-ethylmaleimide, suggesting the involvement of a sulfhydryl compound (87). Rat leukocytes contain NADPH-dependent GSSG reductase and GSH peroxidase, suggesting a sequence in which H_2O_2 oxidizes GSH in the presence of GSH peroxidase; GSSG is in turn reduced by NADPH and GSSG reductase making $NADP^+$ available for HMP activity (Fig. 1) (87). GSH peroxidase differs from MPO in that it is not a hemeprotein and thus is insensitive to cyanide and azide. Human leukocytes contain GSSG reductase (66, 88) and GSH peroxidase has been detected in these cells by some investigators (88, 89) but not by others (66). Baehner, Gilman & Karnovsky (66) suggest that the nonenzymatic oxidation of GSH by H_2O_2 occurs at a rate sufficient to account for the stimulation of HMP activity by H_2O_2. The H_2O_2 required to initiate the sequence may be provided by any of the suggested sources. NADPH oxidase presents a special problem since both NADPH oxidase and GSSG reductase oxidize NADPH. An increase in GSSG reductase activity of guinea pig cells was observed within 15 sec of particle addition, preceding the increase in NADPH oxidase activity (90). A variation of the glutathione sequence was therefore proposed in which NADPH is initially oxidized by GSSG and GSSG reductase, the $NADP^+$ formed stimulates HMP activity and is reduced, and the NADPH is now oxidized by NADPH oxidase with the formation of H_2O_2 which completes the cycle by converting GSH to GSSG (Fig. 1) (90).

INTRALEUKOCYTIC MICROBICIDAL SYSTEMS

The antimicrobial agents of leukocytic origin can be classified into (*a*) products of leukocytic metabolic activity; (*b*) agents with no known enzyme activity; and (*c*) agents with enzyme activity.

PRODUCTS OF LEUKOCYTIC METABOLIC ACTIVITY

Phagocytosis is dependent for energy on glycolysis; it is unaffected by anaerobiasis and is decreased by glycolytic inhibitors (41–43). ATP is rapidly lost during phagocytosis (91), and is reformed predominantly by glycolysis (92). A source of preformed energy may allow some phagocytosis to occur in the absence of active glycolysis (91). The microbicidal activity of leukocytes, however, is depressed by anaerobiasis (93–95), suggesting that it is dependent, at least in part, on the respiratory burst. Of the products of the respiratory burst, acid and H_2O_2 may be microbicidal.

Acid.—Among the consequences of the metabolic burst is a fall in intravacuolar pH presumably owing to increased lactic acid formation (96–99). "Whilst the phagocyte is still living the acid juice which fills the vacuoles or permeates the ingested organisms does not mix with the protoplasm which is always alkaline" (96). The intravacuolar pH has been estimated with the use of indicator dyes to be between 6.0 and 6.5 (99), 4.7 and 5.5 (98), and as low as 3.0 (97). Although the estimation of intracellular pH by these methods is subject to considerable error, the results nevertheless suggest that a considerable acidity way exist within the vacuole. The sensitivity of microorganisms to acid varies considerably. Some, e.g., pneumococci, are very sensitive while others, e.g., lactobacilli, are acidophilic. Other organisms survive a moderate drop in pH although multiplication is impaired. The fall in pH may increase the activity of other microbicidal systems, e.g., granular cationic proteins or MPO.

H_2O_2.—The suggestion that H_2O_2 formed by phagocytizing leukocytes may be microbicidal in situ (45) is supported by a correlation between H_2O_2 production and microbicidal activity in leukocytes treated in a number of ways. Thus, anaerobiasis (94), catalase (94), phenylbutazone (100), hydrocortisone sodium succinate (81), and whole body X irradiation (101) decrease the H_2O_2 production and microbicidal activity of isolated leukocytes. The decrease in microbicidal activity of leukocytes under anaerobic conditions is partially reversed by H_2O_2 (94). The relationship of H_2O_2 to the MPO-mediated antimicrobial system and to the defect in CGD is considered below.

Antimicrobial Agents With no Known Enzyme Activity

Granular cationic proteins.—Heat-stable and cationic antimicrobial agents have been extracted, generally by weak acid, from either intact leukocytes or isolated granules. Hirsch (102, 103) described an antimicrobial agent, phagocytin, in rabbit leukocyte extracts obtained by repeated freezing and thawing of intact cells in buffer at pH 5.6. This preparation had an acid pH optimum and was completely inactivated by heating at 100°C for 30 min. In a later study (104), extraction was performed at a more acid pH (e.g., 0.01 *M* citric acid). The antimicrobial activity was increased as was the range of organism susceptibility; heating for 30 min at 100°C, however, produced only a twofold diminution in activity. Phagocytin is present in the granular fraction and is released and "consumed" in the degranulation process (105). Skarnes & Watson (106) obtained an antibacterial factor, leukin, from rabbit leukocytes by prolonged extraction (12–96 hr) at 37°C with either saline or a saline-lactic acid mixture. The pH generally reached 2.5 to 3.0 in 24 hr. Leukin was not inactivated by heating at 100°C for 90 min. Gram-negative bacteria were resistant to leukin in the initial study; however, in a later study (107), Gram-negative organisms were found to be killed at a more acid pH. The purified factor was an arginine-rich basic protein. Fishman & Silverman (108) obtained a heat-stable bactericidal agent from rat leukocytes by ultrasonic vibration. This agent was present in a fraction which sedimented between 600 and 8500 *g*.

Leukocyte granules are rich in cationic proteins with antimicrobial activity (109). They are extracted by acid, are heat-stable, and can be separated into a number of fractions each with different antimicrobial specificity (110). Histochemical studies on intact cells localized cationic proteins in the primary granule (20, 21); however, citric acid extracts of both A and B particles were bactericidal (18, 19). The presence of a separate enzyme-free granule rich in cationic proteins has been suggested (25). The cationic proteins are released following phagocytosis and coat the ingested organisms (111). Extraction by the procedures employed for phagocytin and leukin preparation would release granular cationic proteins. Leukin, however, was obtained from whole cells by prolonged extraction and may also contain nongranular cationic proteins with antimicrobial activity, e.g., nuclear histones. Phagocytin may contain a heat-labile (enzymatic?) component in addition to the heat-stable cationic proteins.

Lactoferrin.—Lactoferrin, an iron-binding protein present in the specific or secondary granules (24), is bacteriostatic when not fully saturated with iron (112, 113). Its role in the intact cell is unknown.

ANTIMICROBIAL AGENTS WITH ENZYME ACTIVITY

The degradation of intracellular organisms is indicated by morphological changes and by the release of acid-soluble fragments of labeled bacteria into the medium (114, 115). This is presumably a function of the acid hydrolytic enzymes of the cytoplasmic granules; however, there is no evidence that these enzymes are responsible for the death of the organisms. Rather, it is the prevailing view that the microbicidal and digestive processes are distinct and sequential. Of the leukocytic enzymes, only two, lysozyme and MPO, have been implicated in the microbicidal activity of the cell.

Lysozyme.—Lysozyme (muramidase) hydrolyzes the cell wall mucopolysaccharides of certain bacteria, thus rendering them susceptible to osmotic shock. Granulocytes are rich in lysozyme, and the enzyme is present largely in the granular fraction (9, 18, 19). Organisms susceptible to lysis by lysozyme may be killed by this enzyme in situ. However, the number of susceptible organisms is small. Certain Gram-negative organisms, normally insensitive to the lytic action of lysozyme, are made sensitive by pretreatment in a number of ways, e.g., with specific antibody and complement (116) or with ascorbic acid and H_2O_2 (117). Lysozyme may contribute to the death of the organisms under these conditions (117, 118); however, bacterial death usually precedes the action of lysozyme suggesting that the function of this enzyme in most instances is to assist in the digestion of bacteria killed in other ways. The leukocytes of Guernsey and Hereford cattle, which have no lysozyme, kill *Staphylococcus albus, Salmonella typhimurium,* and *Streptococcus pyogenes* normally (119).

Myeloperoxidase.—MPO is present in exceptionally high concentrations (120, 121) in the azurophil, primary or A granules and is released into the phagocytic vacuole during granule lysis (122, 123). Its activity is increased by phagocytosis (65) or by lysis of isolated granules (29, 124). Like the cationic proteins, it is extracted from leukocytes by weak acid (125). It is a basic protein with an isoelectric point greater than 10 (120) and is completely inactivated by heating at 90°C for 10 min (120).

MPO has potent bactericidal (123, 125–131), fungicidal (123, 132, 133), and virucidal (134) activity. H_2O_2 and a cofactor such as iodide (123, 125, 128, 132–134), bromide (123, 125, 128, 132), chloride (123, 125, 128, 130–134), or thiocyanate (126, 127) ions is generally required. Iodide can be replaced by thyroxine or triiodothyronine (128). A microbicidal effect is observed in the absence of added cofactor when the MPO and H_2O_2 concentrations are high (125, 129); the cofactor, however, may be supplied by the microorganism or as an impurity of the other reagents under these conditions. The pH optimum is generally between 4.5–5.0. The source of H_2O_2 in the intact cell may be either leukocytic (see above) or microbial metabolism. When H_2O_2-generating microorganisms are incubated with MPO and a cofactor, auto-inhibition occurs (125, 127). The H_2O_2 formed by one organism also may be utilized for the destruction of a second non-H_2O_2 generating organism by the peroxidase system (123, 135). Studies on CGD (see below) emphasize the importance of microbial H_2O_2 in the microbicidal activity of the intact cell. Of the cofactors, iodide is concentrated by human leukocytes as are thyroxine and triiodothyronine (136). An accelerated turnover of thyroxine and triiodothyronine in patients with bacterial pulmonary infections has been reported (137). Chloride is present in horse (138) and rabbit (139) leukocytes in concentrations considerably higher than required for the MPO-mediated antimicrobial system. Some other, as yet unidentified, cofactor may be operative and certain microorganisms may be oxidized by MPO and H_2O_2 directly without the involvement of an intermediate cofactor.

The MPO-mediated antimicrobial system is inhibited by a number of low molecular weight reducing agents of possible biological significance, e.g., reduced glutathione, NADH, NADPH, cysteine, or ascorbic acid (125, 127, 128). Ascorbic acid also may have a potentiating effect by acting as a H_2O_2-generating system (127). Catalase, by competing with peroxidase for the available H_2O_2, would be expected to be inhibitory and it is under most circumstances. However, when H_2O_2 is maintained at very low levels, catalase, like peroxidase, has antimicrobial activity at acid pH when combined with iodide ions (140). The catalase of horse leukocytes is, in part, particulate (30), whereas guinea pig leukocyte catalase is almost entirely in the soluble fraction (29, 124). Human leukocytes have been reported to contain little or no catalase (141). These differences may reflect species variation; however, studies of the distribution of catalase are complicated by possible contamination with erythrocytes or macrophages with their high catalase contents (141, 142), and by the ability of MPO to convert H_2O_2 to O_2 and H_2O (143).

MPO may exert its effect by catalyzing the conversion of the cofactor from a weak to a strong antimicrobial agent. The cofactor may also stabilize the enzyme (144). When iodide is employed in the cell-free system, iodination of the bacteria occurs and a correlation exists between iodination and bacterial death (128). Chloride (and bromide) stimulate the MPO-catalyzed iodination reaction when the iodide concentration is low (123). Bromination of the bacteria also occurs (Klebanoff, unpublished data). The chloride-stimulated decarboxylation and deamination of amino acids by MPO and H_2O_2 (145) form aldehydes which may have antimicrobial activity (130, 131).

Iodination occurs in the intact leukocyte following phagocytosis (128) and the silver grains are most commonly seen near the surface of the ingested particle (123). Iodination by intact cells is decreased by peroxidase inhibitors such as azide or cyanide (123), and is greatly depressed or absent in leukocytes devoid of MPO and in leukocytes from patients with CGD which are deficient in H_2O_2 (see below). This suggests that the components of the MPO-mediated iodinating (and microbicidal) system are capable of reaction in the intact cell. Do they react, and if so, to what degree do they account for the microbicidal activity of the cell? The decrease in the microbicidal activity of MPO-negative leukocytes (73) is considered below. Azide has a striking inhibitory effect on the microbicidal activity of intact normal leukocytes but has little or no effect on MPO-negative leukocytes (76). The inhibitory effect of azide, therefore, may reflect the contribution of MPO to the total microbicidal activity of the normal cell. If so, it is considerable. It should be emphasized that the iodination reaction, while a measure of the reactivity of MPO in the intact cell, is not necessarily the natural vehicle for the MPO-mediated microbicidal system. Some other cofactor, e.g., chloride, may be primarily involved.

EVALUATION OF LEUKOCYTE FUNCTION

The key laboratory procedure is the measurement of intraleukocytic microbicidal activity. In general, leukocytes isolated by dextran sedimentation are incubated with bacteria (or fungi) and serum under standardized conditions designed to optimize phagocytosis, and the viable cell count is determined after an appropriate interval. Fungicidal activity can be determined by dye exclusion (95). Most methods also measure phagocytic activity, and a defect in intracellular killing is indicated by impaired microbicidal activity associated with normal phagocytosis. Phagocytosis can be estimated by visual examination of stained smears (146) or monolayers (147) for the mean number of particles ingested per phagocyte, the percentage of active phagocytic cells, or the percentage of particles ingested. Phagocytosis also can be estimated by the disappearance of viable bacteria (148) or latex particles (57) from the medium. The cells may be initially removed by centrifugation; however, the decrease in total bacterial count can be employed as a measure of phagocytosis under conditions in which the ingested bacteria are rapidly killed (149). The uptake of radioactive particles (150, 151) or medium (152) by the cells, or the extraction of ingested latex (153, 154) also has been employed as a measure of phagocytosis. Since the presence of viable intracellular organisms in abnormally high numbers is diagnostic of impaired intracellular killing, the cell-associated bacterial count has been determined following the removal of the extracellular fluid by centrifugation (148) or by decantation when monolayers are employed (155). The extracellular bacteria may be destroyed with antibiotics (155, 156).

Nitroblue tetrazolium (NBT) is reduced to a compound with low solubility and an intense blue color. When intact leukocytes from normal persons are incubated with NBT, a small proportion of the cells contain blue inclusions (spontaneous NBT reduction), whereas a much higher percentage stain following the ingestion of particles (phagocytosis-induced NBT reduction). The proportion of cells with blue inclusions can be determined (qualitative NBT reduction test) (157–162) or the dye can be extracted and the color determined spectrophotometrically (quantitative NBT reduction test) (81, 163). A simple screening test has been described (164). In a method described by Gifford & Malawista (165), a large percentage of leukocytes isolated from a drop of whole blood by adherence to glass was converted to large blue "formazan" cells on the addition of NBT in the apparent absence of particle ingestion.

Oxygen consumption by phagocytizing leukocytes can be determined either manometrically or by oxygen electrode. Glucose 1-^{14}C oxidation is generally determined by collection of $^{14}CO_2$ in alkali and measurement in a liquid scintillation counter (166, 167). This procedure is a simple and sensitive measure of the respiratory burst. Methods for the measurement of H_2O_2 formation (45, 51, 52, 81, 142, 166, 167) may measure only a small proportion of the H_2O_2 formed unless H_2O_2 utilization or destruction is inhibited (52). Phagocytosis-induced iodination can be determined radioautographically (128, 168) or by precipitation with trichloroacetic acid (128; Pincus & Klebanoff, unpublished data). MPO can be determined histochemically or following extraction (73). Attempts at quantitation of the histochemical reaction have been made (169, 170). Morphology of circulating leukocytes can be visualized with the electron microscope (26, 171, 172).

SYNDROMES ASSOCIATED WITH IMPAIRED INTRALEUKOCYTIC MICROBICIDAL ACTIVITY

Chronic (fatal) granulomatous disease of childhood (CGD).—The heightened interest in the intraleukocytic parameters of host defense against microbial agents received its stimulus from studies on CGD (5, 6, 173, 174), a condition first described by Berendes, Bridges & Good in 1957 (175) and found 10 years later to be associated with a decrease in intraleukocytic microbicidal activity (176). The early case histories were fairly typical: severe, widespread, and repeated bacterial infections with *Staphylococcus aureus* and a number of Gram-negative pathogens starting in the first year of life and progressing relentlessly until death at an early age. Lipid-filled histiocytes (177, 178) and widespread abscesses and granulomata were found at autopsy. Genetic transmission suggested an X-linked hereditary disease; there was a strong predilection for males, and some female family members had an intermediate defect in leukocyte function (179) and two populations of peripheral leukocytes (157, 168, 179) compatible with the Lyon hypothesis. The suggestion by one group (180, 181) that CGD in its classical variety has sex-modified autosomal recessive inheritance, based on the demonstration of a leukocyte lesion in fathers of male patients with CGD, has stimulated a lively discussion (182–187). The presence of a microbicidal defect in the leukocytes of the father studied by Thompson et al (180) was not confirmed (188).

Neutrophils from patients with CGD have an impaired ability to kill those bacteria generally found in the lesions, e.g., *S. aureus* and certain low-grade Gram-negative pathogens (176), while other bacteria, e.g., streptococci (189), lactobacilli (168), and pneumococci (190) are readily killed. The neutrophils also have decreased virucidal (191) and fungicidal (95, 192, 193) activity and a defect in the microbicidal activity of peripheral monocytes has been demonstrated (194, 195). The phagocytosis-induced stimulation of O_2 consumption (166), glucose 1-C oxidation (53, 166), formate oxidation as a measure of H_2O_2 formation (166, 167), NBT reduction (53, 157, 163, 196) and iodination (168) are greatly depressed. A chromosomal abnormality and a serum factor which impaired lymphocyte transformation was reported in one patient (188).

Intraleukocytic defects similar to those of CGD have been found in patients with atypical histories, suggesting a spectrum of conditions differing in their basic cause. Male patients with no detectable lesion in the leukocytes of the mother (197, 198) may represent non-X-linked disease or a new mutation. The presence of a comparable intraleukocytic defect in female patients (159, 163, 199, 200), particularly siblings (159), suggests the presence of a variant of CGD which is not X-linked. Two female patients with recurrent cold staphylococcal abscesses (Job's syndrome) were described by Davis, Schaller & Wedgwood (201). A third patient considered by Bannatyne, Skowron & Weber (202) to have Job's syndrome, had a leukocyte defect comparable to CGD, suggesting the designation of Job's syndrome as a variant. However, an intraleukocytic defect has not been detected in the two original patients (203). A disease (familial lipochrome histiocytosis) characterized by lipochrome granulation of histiocytes, hyperglobulinemia, splenomegaly, pulmonary infiltration, arthritis, and recurrent infections was described by Ford et al in female siblings (204). Their leukocytes have a defect similar to that of CGD (205). The pigmented histiocytes also are similar in the two conditions (206). A leukocyte lesion almost identical to that seen in CGD was found in a Caucasian female with hemolytic anemia, a leukemoid reaction, severe recurrent infections and a complete absence of leukocyte glucose-6-phosphate dehydrogenase (G6PD) (207). Leukocyte dysfunction is seen only with a severe deficiency of leukocyte G6PD; a patient with 25 percent of normal values had leukocytes with normal metabolic and bactericidal activity (208). The suggestion that the increased susceptibility of males to infection may be owing to an abnormality of G6PD (209, 210) has been questioned (208). A patient with a microbicidal defect against *S. aureus* but not against *Serratia marcescens* (211), and a patient with a temporary microbicidal defect (212) have been described.

The cause of the leukocyte abnormality in CGD has been the subject of some controversy. Early studies revealed the lysozyme and phagocytin activity of leukocyte extracts to be normal as was the cellular MPO activity as estimated histochemically (176). A selective deficiency of granular cationic proteins was suggested (110) but has not yet been demonstrated (213). A number of investigators has reported a decrease in vacuole size (176, 189, 196, 214), suggesting an impairment in degranulation. In other studies the vacuoles of CGD leukocytes could not be distinguished from those of normal cells (122, 158, 190, 200, 215). Incorporation of medium lysolecithin into cellular lecithin, which may be important for membrane formation, is increased during phagocytosis by CGD as well as by normal leukocytes (215). Granule-associated enzymes are released by CGD cells following phagocytosis as measured either histochemically (122, 158, 190, 196) or by the enzyme activity of the nongranular fraction (122), indicating that degranulation does occur; what remains uncertain is whether the rate of degranulation is decreased. It is questionable whether a decreased rate of degranulation can be the sole cause of the profound defect in microbicidal capacity unless degranulation is very substantially delayed.

The importance of the decrease in H_2O_2 formation by CGD leukocytes is emphasized by the improvement in leukocyte function on the introduction of a H_2O_2-generating system into the cell. Organisms, such as streptococci (189, 216), lactobacilli (168), and pneumococci (190, 216) which are killed well by CGD leukocytes, generate H_2O_2 and thus their susceptibility to the intraleukocytic microbicidal systems may be owing to the replacement of a defective leukocytic H_2O_2 generating system with H_2O_2 of microbial origin (168). The defect in CGD leukocytes also may be partially reversed by H_2O_2-producing drugs (38, 217) or by the introduction into the cell of latex particles coated with glucose oxidase (167, 218). The killing of *Lactobacillus acidophilus* by CGD leukocytes is inhibited by azide (76), cyanide (76), and methimazole (168), suggesting the involvement of the MPO system. CGD leukocytes are considerably more sensitive than normal leukocytes to methimazole.

The enzymic basis for the defect in CGD is controversial. Baehner & Karnovsky (219) demonstrated a deficiency in NADH oxidase in one female and four male patients with CGD; Holmes, Sater & Good (personal communication), however, could not detect a deficiency of this enzyme in their patients. The leukocytes of two female patients with CGD were reported by Holmes et al. (88) to be deficient in GSH peroxidase; leukocytes of male patients contained normal amounts. The G6PD of leukocytes of male patients with CGD was reported to be more labile than that of normal cells (220) and this has been confirmed (88). The importance of G6PD in the microbicidal activity of the leukocyte has been emphasized by the presence of a functional defect in a patient with absent leukocyte G6PD (207).

Myeloperoxidase deficiency.—MPO deficiency may involve all or a percentage of the neutrophils; MPO may be undetectable or decreased in amount, and the deficiency may be permanent or temporary. The deficiency may reflect a generalized lysosomal lesion or a specific enzyme defect.

Five patients (three families) have been described with a complete and presumably permanent absence of peroxidase from all neutrophils and monocytes (73, 221–223). Genetic studies suggested autosomal recessive inheritance. Of these patients, only one was reported to have an increased susceptibility to infection. This patient, who reached adulthood without serious problems, was an adult-onset diabetic with systemic candida infections (73). His leukocytes were studied extensively by Lehrer, Cline et al, and a functional defect found which differed significantly from that of CGD. The phagocytosis-induced increase in O_2 consumption and glucose 1-C oxidation was within the normal range (73); iodination, however, was markedly decreased (Pincus & Klebanoff, unpublished data). His leukocytes were defective in fungicidal (73, 193) and bactericidal (73, 224) activity. A marked deficiency of MPO is required before a microbicidal defect can be demonstrated. The children of the MPO-negative patient contained 30 percent of normal levels of MPO and exhibited normal leukocyte microbicidal activity by the assay employed (73). Azide had little or no effect on the microbicidal activity of MPO-negative leukocytes in contrast to the striking inhibition of normal cells (76); indeed, the microbicidal activity of azide-treated MPO-negative leukocytes was greater than that of similarly treated normal cells (76). This suggests the possibility of an adaptation in which the azide-insensitive (non-MPO) antimicrobial systems increase in activity and thus compensate, in part, for the loss of azide-sensitive microbicidal systems. The nature of the adaptation is unknown. H_2O_2 accumulates when utilization is decreased by agents such as cyanide which inhibit MPO as well as other hemeproteins (52). One possibility is that H_2O_2 accumulates in MPO-deficient leukocytes to levels which are microbicidal in the absence of MPO (76).

Isolated cases with an absence of MPO-positive granules in some or all of the neutrophils have been reported by others (169, 225–227). These patients were severely ill and in some the peroxidase reaction became positive when the patient recovered (see *Infection* below).

Chediak-Higashi syndrome.—The Chediak-Higashi syndrome (228) has been described in man, mink, cattle, and mice. The disease in man occurs in childhood, is characterized by partial albinism, photophobia, frequent and severe pyogenic infections, hepatosplenomegaly, and giant granules (lysosomes) in leukocytes as well as in other cells. The cause of the increased susceptibility to infection is unknown. Granulocytopenia owing to increased destruction of granulocytes (229) is a common finding and decreased leukotaxis has been reported (230). The lysosomal abnormality has stimulated interest in the possibility of an intracellular microbicidal defect; however, attempts to demonstrate one have been inconclusive (228, 231, 232). The designation of Chediak-Higashi syndrome as a neutrophil dysfunction syndrome associated with an intraleukocytic microbicidal defect must await further study.

Newborn infants.—Newborn, particularly premature, infants have an increased susceptibility to infection. Reports of leukocyte abnormalities are conflicting. Neutrophils from full-term neonates have been reported to have decreased phagocytic activity owing either to decreased serum opsonic capacity (233, 234) or to cellular factors (234). Other investigators report the phagocytic activity of leukocytes from full-term neonates to be normal (235) and that of premature infants to be increased (236). Leukocytes of premature infants (236) and some full-term infants less than 12 hrs old (235) have been reported to have an intraleukocytic microbicidal defect; other investigators, however, could not detect a defect in full-term neonates (233, 237). Leukocytes from full-term neonates (and their mothers) were reported to have increased spontaneous metabolic activity and a normal phagocytosis-induced increase in O_2 consumption and glucose 1-C oxidation (237); other investigators, however, could not detect a phagocytosis-induced increase of glucose 1-C oxidation in infants less than 12 hr of age (235). Spontaneous nitroblue tetrazolium reduction by leukocytes from both full-term (162, 237–239) and premature (239) neonates is high; this elevation is not observed in premature neonates with bacterial infection (239).

Thermal injury.—Patients with severe thermal injury develop an intracellular killing defect (240, 241). These patients are very susceptible to septic complications. Thermal injury is associated with a decrease in the leukocytic content of three granular enzymes, β-glucuronidase, acid phosphatase, and lysozyme (242), suggesting lysosome depletion. Cyclic variation in microbicidal activity may affect neutrophil function in normal persons and in patients with burns and other disorders (240, 241).

Infection.—Leukocytes from patients with severe infection may be abnormal. An increased spontaneous reduction of NBT is observed in bacterial (160, 162, 243) but not in viral disease (160). Graham (169) reported a gradual decrease and in some instances a complete disappearance of MPO-positive granules from the leukocytes of patients with uncontrolled bacterial infection. This lesion may represent general granule failure rather than specific MPO deficiency. Iron deficiency however may contribute to a decrease in MPO levels. Prolonged infection is associated with mild anemia owing, in part, to restricted iron supply as a result of a block in iron release from reticuloendothelial cells (244). Iron deficiency anemia is associated in some patients with a significant decrease in MPO (245, 246). A decrease in the microbicidal capacity of the leukocytes of some patients with infection has been reported (240, 247). Since infection is the primary consequence of the leukocyte dysfunction syndromes, an effect of infection per se on the intraleukocytic microbicidal systems would be superimposed on that of the primary lesion. Toxic granulation, which is found in patients with severe infection or other "toxemias" (248), has been noted in the leukocytes of a patient with CGD (249).

Exogenous agents.—A number of exogenous agents of possible physiological, pharmacological or pathological significance may affect the metabolic and microbicidal activity of leukocytes. These include bacterial products such as staphylococcal leukocidin (250), streptolysin (33, 251–253), and endotoxin (254); vitamins such as vitamin A (33, 255); hormones such as corticosteroids (36, 81, 256), etiocholanolone (36) and estrogens (36, 257); and pharmacological agents such as salicylates (258, 259), chloroquine (36, 260), phenylbutazone (100), colchicine (261, 262), levorphanol (263), polyene antifungal antibiotics (36), chloramphenicol (264, 265), methimazole (168), and propylthiouracil (266). In some instances, large doses are required and their role in vivo is uncertain. Some endocrine and pharmacological agents may influence granulocyte activity through the regulation of intracellular cycle AMP levels (267, 268).

SUMMARY

The neutrophil is highly specialized for its primary function, the phagocytosis and destruction of microorganisms. A number of antimicrobial systems exist in the cell and the one which predominates may vary with the microorganism and the metabolic state of the leukocyte. Their level and variety give the leukocyte an overkill capacity and thus a reserve against the decrease or loss of one of the antimicrobial systems. Thus, a primary deficiency of cationic protein, lysozyme, or myeloperoxidase may leave other antimicrobial systems intact, and the leukocytes may function adequately because of their microbicidal reserve unless greatly stressed. The efficiency of one antimicrobial system may increase to compensate for the decrease in another. The microbicidal deficiency may be widespread. Thus, a primary enzyme defect involving a metabolic pathway may decrease both acid and H_2O_2 production and thus the efficiency of the MPO system. If decreased degranulation is a consequence, cationic protein, lysozyme, lactoferrin, and myeloperoxidase also may be less readily available. Similarly, a primary degranulation defect will affect many other parameters of the leukocyte response. The result is more severe disease.

As the intraleukocytic microbicidal mechanisms are elucidated and their relationship to the microbicidal activity of the cell established, physiological and pathological variations will become apparent. Ultimately, a classification of conditions associated with intraleukocytic microbicidal defects will evolve which takes into account the basic molecular lesion. Leukocyte deficiencies of acid, granular cationic protein, lysozyme, or lactoferrin have not yet been detected in man. There appears, however, to be a family of conditions in which the decreased microbicidal activity is associated with H_2O_2 deficiency. The cause of the H_2O_2 deficiency may vary; theoretically, a deficiency could result from decreased O_2 or substrate supply, from a defect in the mechanism triggering the respiratory burst, from a defect in any one of a number of enzymes required for the metabolic sequence leading to H_2O_2 formation, or from increased H_2O_2 destruction or utilization by nonmicrobicidal systems. This lesion is characterized by its reversal by H_2O_2-generating systems including bacteria, and by the decrease in other H_2O_2-dependent parameters of leukocytic activity, e.g., iodination and glucose 1-C oxidation. Decreased O_2 consumption and NBT reduction also may be present. CGD is the prototype of the H_2O_2-deficiency syndrome. Another leukocytic lesion leading to impaired microbicidal capacity is MPO deficiency. The functional defect is less severe than that associated with H_2O_2 deficiency. This lesion is characterized by a decrease in MPO and by decreased iodination. Microbicidal activity is azide-insensitive. The metabolic burst is intact. Finally, there may be a microbicidal defect in which a decrease in granule numbers or an abnormality in either granule structure or the degranulation process is the primary lesion. A number of rare genetic abnormalities provide striking evidence of the importance of intact intraleukocytic microbicidal systems in the host defense. The role of more subtle, acquired microbicidal defects in the initiation and course of infection is a subject of increasing interest.

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KININS IN HUMAN DISEASE

7004

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The kinins are a group of straight-chain polypeptides which resemble bradykinin in structure and pharmacological activity (1). Besides bradykinin, the group includes kallidin, methionyl-lysyl-bradykinin, glycyl-bradykinin and phyllokinin. These polypeptides are formed by enzymic action on $\alpha 2$-globulins in plasma, the term kininogen being applied to the plasma substrate, and kininogenase to the enzyme responsible for the production of kinin. The breakdown of kinins to inactive peptides is effected, in turn, by enzymes collectively known as kininases (Fig. 1). The prototype of the kinin group is bradykinin, a nonapeptide with the structure indicated in Figure 2; the other members of the group all exhibit this nonapeptide sequence and differ only in having additional amino acid residues at the N- or C-terminal.

$$\text{KININOGEN} \xrightarrow{\text{Kininogenase}} \text{KININ} \xrightarrow{\text{Kininase}} \begin{array}{c}\text{INACTIVE}\\ \text{PEPTIDES}\end{array}$$

Figure 1. Simplified scheme of the formation and breakdown of kinins.

In view of the considerable clinical interest which the kinins have evoked, it is rather surprising how much our knowledge of them should have arisen mainly from various investigations of "laboratory artifacts" (2). For example, an investigation of a vasodepressor substance in urine (3–5) resulted in the discovery of kallikrein; while an observation that a potent permeability factor (PF) was activated when guinea pig plasma was diluted (PF/dil) (6–9) opened the way for the identification of activators of the inert precursor of kallikrein. The discovery of bradykinin (10) itself came from the finding that trypsin or snake venom released a peptide-like substance from a plasma substrate, the name "bradykinin"[1] referring to the ability of the peptide to induce slow contraction of guinea pig ileum in vitro. Its identification as a nonapeptide (11–14) opened a new era in pharmacology.

After this breakthrough, the pace of investigation of kinins quickened

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[1] Kinesis = movement.

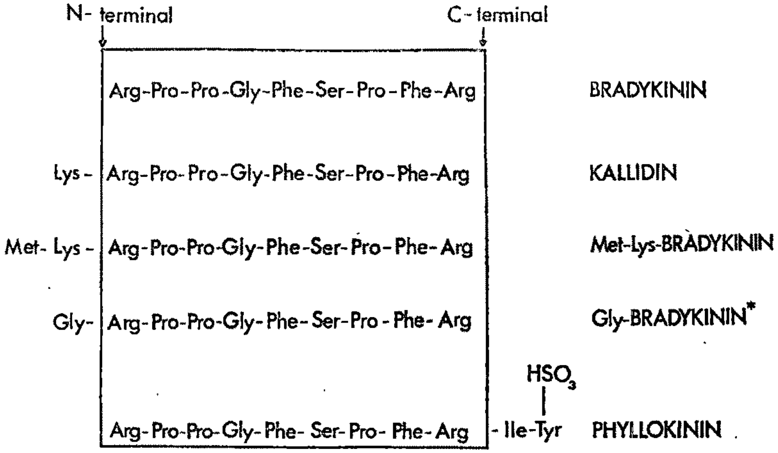
N- terminal
C- terminal
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
BRADYKININ
Lys-
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
KALLIDIN
Met- Lys-
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Met-Lys-BRADYKININ
Gly-
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Gly-BRADYKININ*
HSO3
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
-Ile-Tyr
PHYLLOKININ

FIGURE 2. Amino acid sequence of kinins. Each contains the nonapeptide sequence of bradykinin, as indicated within the block. Bradykinin, kallidin, and methionyl-lysyl-bradykinin are released, respectively, by trypsin, kallikrein, and acidification from a substrate in mammalian plasma or serum. Of these three kinins, the first two have been prepared from human and other mammalian plasma, the third only from bovine plasma. Glycine-bradykinin (see Reference 1) is released by trypsin from an octadeca-peptide in wasp venom. Phyllokinin has been isolated from amphibian skin. Redrawn and modified from Kellermeyer & Graham (98) and Schachter (38).

* Alternative name = gly-kallidin.

rapidly. Pierce and Webster (15, 16) soon reported the release by urinary kallikrein of two kinins from plasma globulins—the first being the nonapeptide, bradykinin; the second, a decapeptide which differed from bradykinin only in having an additional N-terminal lysine residue. Lysyl-bradykinin was subsequently synthesized by Nicolaides et al (17) and identified as kallidin (Fig. 2). Met-lys-bradykinin was next isolated from acidified preparations of bovine pseudoglobulin (18, 19), and its structure confirmed synthetically (20, 21). Bradykinin, kallidin, and met-lys-bradykinin are therefore all released from human and bovine kininogens (22). On the other hand, gly-bradykinin (Fig. 2) is obtained by tryptic digestion of an octadecapeptide in wasp venom (23, 24), while phyllokinin is isolable from amphibian skin (25, 26).

Clinical interest in the kinins has been evoked (*a*) by their outstanding pharmacological activity, particularly as vasodilators and as factors increasing vascular permeability; (*b*) by a quartet of effects which mimic the main features of the inflammatory response—viz., vasodilatation, increased vascular permeability, local emigration of leukocytes, and pain; (*c*) by their occurrence in significant amounts in a wide range of human diseases. But before discussing the assessment of the importance of kinins in human medicine, I shall briefly summarize present knowledge of their formation, breakdown, and pharmacological properties.

Kinin formation.—Kallikreins: Enzymes capable of forming kinins are collectively termed kininogenases and include the kallikreins, trypsin, and pepsin, as well as proteases in snake venoms and bacterial products. All are proteolytic and esterolytic enzymes, of which most share with trypsin the ability to hydrolyze peptide bonds in which the carboxyl group is contributed by arginine. Of the kininogenases, the kallikreins have greatest hydrolytic activity and substrate specificity. They are widely distributed in the body and can be divided into (*a*) tissue and (*b*) plasma kallikreins. Various tissues, particularly of glandular organs, contain one or more (27, 28) kallikreins which are often secreted in an active form: e.g., in saliva (salivary gland, 29); pancreatic juice (pancreas, 30); sweat (skin, 31); tears (32); feces (small and large bowel, 33, 34); and urine (kidney, 33, 35, 36). Although the tissue kallikreins (also referred to as "glandular" kallikreins) are closely related, they nevertheless are individually distinct. For example, the kallikreins from hog pancreas, submaxillary gland, and urine have molecular weights within the range 33,000 to 36,000 (27, 36), but each differs in composition, physicochemical and immunological characteristics (see Webster, 37; Schachter, 38). The same comments also apply to tissue kallikreins from human pancreas and urine (37).

Compared with the tissue kallikreins, plasma kallikrein is a larger molecule (mol wt = 97,000; 39), which differs physicochemically and immunologically (37, 40, 41), and is probably elaborated as an inert precursor (prekallikrein)[2] by the liver (34, 42). There is evidence, however, that plasma probably contains two kallikreins (see Webster, 37).

Activation of Pre-Kallikrein:[2] The tissue kallikreins have been isolated in subcellular particles in the submaxillary gland (43, 44) and kidney (35), suggesting that these enzymes are stored in the corresponding tissues in an active form. In other tissues, kallikrein exists as an inert precursor for which the mechanism of activation has not been identified. But for human plasma kallikrein, activation involves a series of enzymes which are sequentially converted from pre-enzyme to enzyme, with the latter successively activating the next in the series (Fig. 3) and activation being initiated by Hageman factor (factor XII) which is better known for its role in blood clotting (45, 46). Webster (37) has isolated five kininogenases from human plasma of which Enzyme I is kallikrein and IV is Hageman factor. The order of activation of Enzymes V, III, and II has not been finalized, so that the proposed order indicated in Figure 3 remains tentative (47). However, it seems likely that enzyme II corresponds to PF/dil—i.e., the plasma permeability factor which is activated by dilution (7, 48).

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[2] See nomenclature recommended in Reference 1.

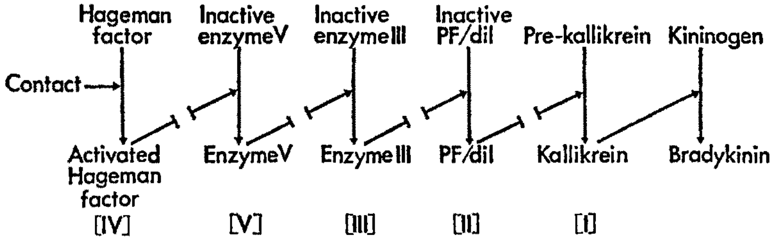

FIGURE 3. The activation of human plasma kallikrein. Redrawn from Webster (47). The Roman numerals in brackets refer to the kininogenases isolated by Webster (37, 47).

Kallikrein Antagonists: These antagonists occur in various animal and plant tissues (49), the best known being "Trasylol," a polyvalent protease inhibitor (mol wt = 6511) which occurs in the parotid, pancreas, and lung of cattle. Other kallikrein antagonists occur in the plasma and pancreas of man, as well as in the pancreas of other species (see Schachter, 38).

Kininogens: Early studies (50) suggested that the plasma kininogens comprised two substrates associated with $\alpha 2$-globulins and corresponding respectively to the decapeptide, kallidin, and the nonapeptide, bradykinin. But it now seems that human plasma contains at least three classes of kininogens (47) which are produced by the liver (51). The first class consists of two kininogens, I and II, which are glycoproteins (Fig. 4) with a molecular weight of about 50,000 (52, 53) and represent the main kininogen con-

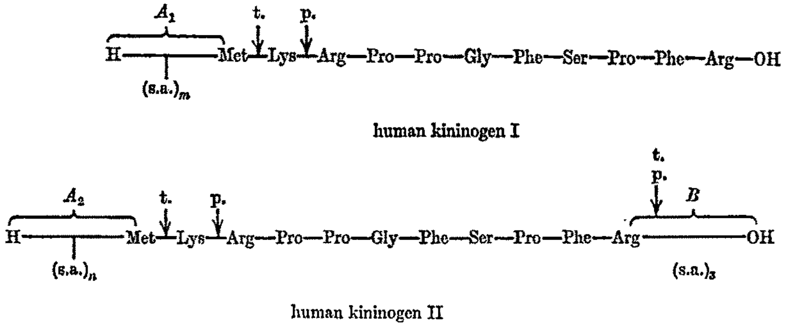
A_1
t.
p.
H—Met—Lys—Arg—Pro—Pro—Gly—Phe—Ser—Pro—Phe—Arg—OH
$(s.a.)_m$
human kininogen I
A_2
t.
p.
t.
p.
B
H—Met—Lys—Arg—Pro—Pro—Gly—Phe—Ser—Pro—Phe—Arg—OH
$(s.a.)_n$
$(s.a.)_3$
human kininogen II

FIGURE 4. Structure of low molecular weight kininogens and the sites of attack by tissue (t) and plasma (p) kininogenases. The kinin chain is attached at its N-terminal end to large fragments, A1 or A2 (mol wt approximately 50,000). The C-terminal end of kininogen I is completed by kinin, and in kininogen II by an additional small fragment B. The fragments A1, A2, and B carry sialic acid residues (s.a.). Reproduced from Eisen (53a) by courtesy of the Editor of the *Proceedings of the Royal Society of London.*

tent of plasma. Kininogen I contains met-lys-bradykinin as the C-terminal sequence, whereas kininogen II contains the same sequence in the middle of the chain (54). These low molecular weight (LMW) kininogens yield kallidin when treated with human urinary kallikrein, and bradykinin when treated with trypsin (47). The other two classes of kininogens have high molecular weight (HMW) and include two groups, a and b. HMW-a kininogens have a molecular weight of about 200,000 and are therefore too large to occur normally in lymph. They yield kallidin when treated with human urinary kallikrein, but bradykinin when treated with human plasma kallikrein or trypsin. In most species (but excluding the guinea pig), HMW-a kininogens therefore represent the only substrate for plasma kallikrein. The HMW-b kininogens have been recognized only recently (55) and are not yet characterized. However, they seem to be hydrolyzed by trypsin alone, and to yield kinins which differ from those obtained from LMW and HMW-a kininogens.

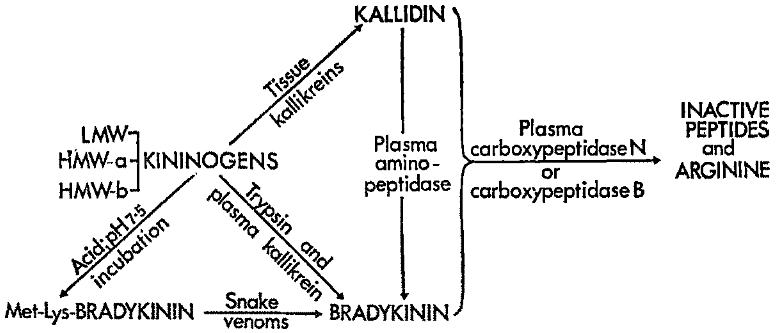

FIGURE 5. Diagrammatic summary of the inter-relation between kininogens, kinins, and their final breakdown products. Adapted from Pierce (53) and Webster (47).

In summary, kallidin is released by tissue kallikreins from both LMW and HMW-a kininogens, whereas bradykinin is formed by the action of plasma kallikrein on HMW-a kininogen, and of trypsin on both LMW and HMW-a kininogens. Nevertheless, the statement that plasma kallikrein forms bradykinin is only applicable when the enzyme is activated by contact with glass (54); when activated by acidification, plasma kallikrein forms met-lys-bradykinin (19; see Fig. 5). Aminopeptidases in blood probably convert met-lys- and lys-bradykinin (kallidin) to bradykinin (Fig. 5), but whether there are specific enzymes for the removal of each of the met-lys and lys moieties has not been established.

Kinin breakdown.—Kinins have a half-life in circulating blood of <15 sec (56), being hydrolyzed by kininases to inactive peptides. Two such kininases have been identified in human plasma. The first, carboxypeptidase N,

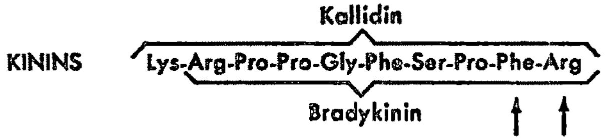

FIGURE 6. Breakdown of kinins by kininases in human plasma. The arrows illustrate the cleavage of the C-terminal arginine by carboxypeptidase N, and the cleavage of the Pro-Phe bond by another peptidase. Reproduced from Webster (47) by courtesy of the Editor of *Cellular and Humoral Mechanisms in Anaphylaxis and Allergy.*

splits off the C-terminal arginine residue from bradykinin and kallidin (57), whereas the second is a peptidase which hydrolyzes the Pro^7–Phe^8 bond (58) (see Fig. 6). Both kininases are distinct from the carboxypeptidases A and B of the pancreas (58, 59). In addition, human plasma contains one, possibly two, aminopeptidases (38) that rapidly convert kallidin to bradykinin, with further hydrolysis then being affected by plasma carboxypeptidases (Fig. 5). Of purified preparations of kininases, carboxypeptidase B from hog pancreas has highest activity in hydrolyzing kinins both in vitro and in vivo (60). Large doses of carboxypeptidase decrease the hypotension induced by kinins and kallikrein, but have less effect on the permeability response evoked by the intracutaneous injection of these substances (61). Tests with the blood-bathed organ technique indicate that circulating kinins are depleted more effectively by the lungs than by the peptidases in plasma (56). Bradykinin is also inactivated by cathepsins in the spleen (62) and kidney (63), and kininases have been demonstrated in epithelial cells throughout the digestive tract (64).

Pharmacological activity.—Pharmacologically, the kinins have outstanding activity as vasodilators of most vessels in the body and therefore cause hypotension. On the other hand, pulmonary vessels may be constricted, with a resultant rise in pulmonary arterial pressure, particularly in cases of chronic bronchitis (65); pulmonary veins, however, may go into spasm (66). The kinins also have outstanding activity as permeability factors (PFs) (67–69), both the permeability and vasoactive effects being obtained with concentrations of only 10^{-9}–$10^{-7}M$. Yet another response obtained with bradykinin injected intracutaneously is the local emigration of neutrophil leukocytes, although the effective concentration of kinin now needs to exceed $10^{-6}M$ (70). At concentrations as low as $10^{-7}M$, bradykinin causes pain when applied to the base of a blister (68), or injected into human skin (71), the brachial artery (72), and peritoneal cavity (73). Apart from their vasomotor effects, kinins (10^{-10}–$10^{-8}M$) induce contraction of smooth muscle in vitro, e.g., of rat uterus, guinea pig ileum or bronchus, but cause relaxation of rat duodenum (68).

KININS IN HUMAN DISEASE

The occurrence of kinins in tissues affected by a wide range of diseases has naturally evoked considerable interest in their corresponding pathogenic role. However, the mere presence of relevant kinins clearly does not warrant the conclusion that they are prime causal factors of these diseases, although in some cases at least, the evidence suggests that the association has a meaningful relation. Kinins have been claimed to play a possible role in the following groups of conditions:

Group I: Conditions associated with vasodilatation, hypotension, or shock:
1. Carcinoid and related syndromes.
2. Shock.

Group II: Other conditions involving the vascular system of the newborn and adult.
Group III: Conditions exhibiting inflammation.
Group IV: Noninflammatory edema.
Group V: Various other diseases.

CONDITIONS ASSOCIATED WITH VASODILATATION, HYPOTENSION, OR SHOCK

Carcinoid and related syndromes: carcinoid syndrome.—Carcinoid tumors arise from argentaffin cells in the gastrointestinal mucosa. Sixty percent occur in the appendix and rarely give rise to metastases. Of the remainder, about two-thirds occur in the small intestine, particularly the ileum. The remaining third mainly involve the stomach or rectum, although occasional tumors are found in the colon, biliary tree, or pancreas (74). Of the extra-appendiceal tumors, about 38 percent are malignant and metastasize to the regional lymph nodes, liver, lung, and bones (75).

Cases of carcinoid tumor which metastasize, particularly to the liver, often exhibit a syndrome which includes vasodilatation and hypotension, diarrhea and intestinal malabsorption, intermittent attacks of bronchoconstriction, and stenosis of the valves of the right side of the heart (particularly the pulmonary valve). The flushing is usually transient, occasionally permanent, involves the face and neck, and has a cyanotic tint. Although the syndrome is generally restricted to cases with metastases, it may be associated with a benign tumor which is relatively large.

Carcinoid tumors have been demonstrated to produce catecholamines, histamine, and 5-hydroxytryptophan. The last is converted by the corresponding decarboxylase to 5-hydroxytryptamine (5-HT) which, in turn, is excreted in the urine as 5-hydroxyindoleacetic acid (5-HIAA). Excessive production of 5-HT is well established in carcinoid syndrome and markedly elevated levels of plasma 5-HT and urinary 5-HIAA are usually diagnostic of the condition (76, 77).

The overproduction of 5-HT probably explains the occurrence of diarrhea and malabsorption (78). On the other hand, several studies have indicated little or no correlation between flushing and the level of plasma 5-HT

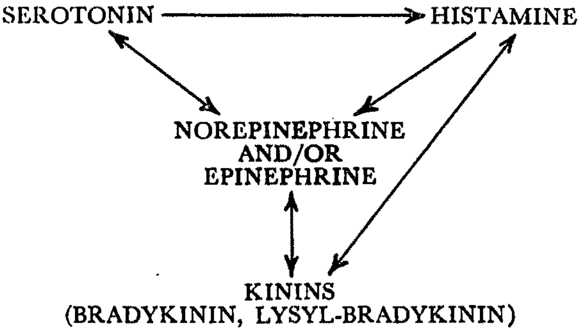
SEROTONIN
HISTAMINE
NOREPINEPHRINE AND/OR EPINEPHRINE
KININS (BRADYKININ, LYSYL-BRADYKININ)

FIGURE 7. Diagrammatic summary of the inter-related release of kinins and amines. A substance at the head of an arrow can be released by the substance at the other end of the arrow. In some cases, release is bilateral. Reproduced from Melmon & Cline (89) by courtesy of the Editor of the *American Journal of Medicine.*

(79–82). Instead, the flushing seems to be associated with the production by these tumors of kallikrein which, in turn, liberates kallidin from plasma kininogen (81–84), the kallidin then being rapidly converted to bradykinin by an aminopeptidase in plasma. Both kinins are powerful vasodilators and occur in highest plasma concentrations at the time of flushing (85).

Carcinoid flushing is readily provoked by intravenous epinephrine. In 11 cases (86) so investigated, 2 initially had high levels of kinin; when flushing was induced, 5 had markedly elevated levels (25 μg kinin/100 ml hepatic venous blood) and 4 others had levels of 9–24 μg/100 ml. In normal subjects, comparable levels of bradykinin produce vasodilatation and transient hypotension (65, 87). However, the remaining 2 of the above 11 cases had kinin levels in the range (0.1–7.9 μg/100 ml) which was normal for control cases without carcinoid, both before and after epinephrine (86). In 5 of 6 of the above cases, the hepatic metastases contained kallikrein (86), with the highest concentrations occurring in 2 cases exhibiting severe flushes and hypotension, whereas none was isolated from a tumor in another patient without flushes. In the 5 cases yielding kallikrein, the level was increased 2–8 times by giving intravenous epinephrine.

Carcinoid tumors are therefore associated with the release of various vaso-active factors for which the release cannot be consistently correlated with the corresponding clinical manifestations; some tumors excrete no 5-HT, while with others there is no increase of plasma kinin. Epinephrine induction of the syndrome seems to involve alpha-receptors, because flushing is reduced by alpha-adrenergic antagonists such as phentolamine and phenoxybenzamine. But not all activators of alpha-receptors will induce flushing, and even beta-adrenergic antagonists (e.g., propanol) decrease the severity and duration of spontaneous flushing (88). As Figure 7 indicates, the pharmacological substances identified in the carcinoid syndrome have a complex inter-relation and the clinical features of the syndrome probably represent the inter-related effects of several vasoactive agents (89).

"Dumping" syndrome.—Abdominal and vasomotor symptoms are common during convalescence from gastrectomy, but persist in about 5 percent. The vasomotor symptoms in such cases of the "dumping syndrome" include flushing, which is associated with elevated levels of plasma kinin and indistinguishable from the flushing in cases with carcinoid. Flushing can be provoked by intravenous epinephrine or by drinking water containing glucose; it is accompanied by hypotension, associated with markedly elevated levels of free plasma kinin and a corresponding decrease of plasma kininogen (90, 91).

Noncarcinoid neoplasms.—A typical carcinoid syndrome may also be associated with the same type of tumor arising in the bronchus—in fact, the flushing is often more intense and particularly prolonged (92). But, apart from carcinoids of the respiratory and gastrointestinal tracts, the syndrome is also exhibited by patients with noncarcinoid neoplasms in various sites—although the flushing is bright red rather than having the cyanotic tint associated with carcinoid. Of 16 such neoplasms listed by Sommers (93), half were "oat-cell" carcinomas of the lung, while the rest included carcinoma of the thyroid, and retroperitoneal neuroblastoma. Besides flushing, the patients regularly had diarrhea. Of seven cases which were investigated, six had elevated levels of plasma 5-HT and urinary 5-HIAA, but only one exhibited an increase of plasma kinin. In two cases, flushing could not be provoked with epinephrine. Such pharmacological features are usually associated with tissues containing argentaffin cells and arising from the foregut (94), but none of these noncarcinoid neoplasms were argentaffin.

Shock.—The similarity of the hemodynamic effects induced by intravenous bradykinin (10, 95) with those of various types of shock has prompted studies of the kinin and kininogen levels in shock induced by endotoxin (96, 97, 99), anaphylaxis (47, 100), hemorrhage (101), the application of tourniquets (96), and the transfusion of plasma products (102).

Endotoxin shock.—The early stages of the vascular collapse in rhesus monkeys given intravenous endotoxin are accompanied by an increased plasma level of kinin and decreased kininogen (96). At later stages, however, when peripheral resistance and cardiac output have progressively decreased, there is no accompanying elevation of plasma kinin. The kinin-forming system may therefore be involved in the early but not the late stages. Such a role for kinins gains some support from other laboratory findings which indicate two possible mechanisms by which endotoxin might activate the kinin-forming system: (*a*) by the formation of endotoxin-antibody complexes, as judged by the ability of antigen-antibody complexes to induce the release of kinin (103, 104); or (*b*) by the interaction of endotoxin with circulating granulocytes (96). Granulocytes contain enzymes which both liberate and destroy kinins (105), but in endotoxin shock, plasma kininase and lysosomal enzymatic activity lag behind the maximal release of plasma kinin so that granulocytes are probably not responsible for an early release of kinin in this condition (96, 98). As Kellermeyer (98) points out, the infusion of kallikrein itself does not lead to fatal shock in dogs, while at the stage of irreversible shock there is only a slight decrease of kininogen (106).

Anaphylactic shock.—As early as 1950, Beraldo (107) observed that anaphylactic shock was accompanied by kinin-like activity in the plasma, although the level of kinin seemed unrelated to the severity of the shock. Considerable evidence has subsequently indicated the activation of the kinin system in experimental anaphylactic shock in the guinea pig, rabbit, rat, and dog (108, 109). In addition, kallikrein is released from guinea pig lung by anaphylaxis in vitro (110, 111). Nevertheless, the contribution of kinins in anaphylactic shock remains uncertain (47). Kinins and histamine both seem to play a role in anaphylaxis (111a) although their effects are probably confined to the early stages. Bradykinin can induce hypotension and is increased in the plasma during various hypotensive stages, but its primary role remains to be established (98).

Reactions to plasma products.—The transfusion of a fraction of human plasma rich in fibrinogen and anti-hemophiliac globulin may be complicated by a severe reaction (102). The fraction contains activated plasma kallikrein and relatively high levels of the esterase of the first component of complement (C′1) (112), the latter being activated by the fractionation procedure involving the use of ether (113). Sufficient kallikrein is activated to account for the formation of large doses of bradykinin. Another example of the possible role of kinins in reactions to transfusion comes from experiments in dogs given homologous blood by exchange or cardiopulmonary bypass techniques (114–116). The consequent arterial hypotension and portal hypertension can be obviated by preventing contact of the blood with glass (116), a result which suggests that the Hageman factor-kallikrein-kinin system may be involved.

OTHER EFFECTS ON THE VASCULAR SYSTEM OF THE NEWBORN AND ADULT

Melmon and his colleagues (117) have suggested that plasma pre-kallikrein is activated at birth with the resultant kinins being important in the circulatory changes in the newborn, whereby the fetal circulatory system becomes converted to that of the adult. Kinins are potent in vitro vasoconstrictors of the umbilical artery and vein of the fetus in man and sheep, as well as of the ductus arteriosus of the fetus of cattle and sheep (117, 118). Such effects of kinins are minimal at oxygen tensions obtained in the fetus, but maximal at those in the newborn. Moreover, the level of kinin in umbilical cord blood is significantly higher than the plasma level in adults. With the additional observation that bradykinin induces dilatation in vivo of the pulmonary artery of the fetal lamb, the overall results (117, 118) certainly warrant further investigation of the importance of kinins in the newborn. On the other hand, reports that kinin levels are elevated in childbirth (119) may prove more relevant to tissue damage during labor than to pregnancy.

In the adult, the vasodilator effects of kinins are reported to occur in tissues other than salivary gland. In dogs, direct renal infusion of bradykinin is reported to increase blood flow in the kidney, as well as the glomerular filtration rate (120). Other work with kallidin suggests, however, that enhancement of renal blood flow is not accompanied by increase in the glomerular filtration rate (121). Nevertheless, both studies revealed an increased urinary excretion of sodium. In normal human subjects, infusion of bradykinin is reported to enhance renal blood flow, to decrease glomerular filtration rate, and to increase urinary excretion of sodium (122) so that there are at least some grounds for suspecting that kinins may be concerned in the regulation of renal function. Kinin-induced vasodilatation has also been claimed to play a role in muscular contraction. Direct perfusion of kallidin into in vitro preparations of muscle markedly decreases vascular resistance (123) which is suppressed, in turn, by simultaneous perfusion of carboxypeptidase B. On the other hand, the vasodilatation induced by muscular contraction, by release of arterial occlusion, or by stimulation of lumbar cholinergic sympathetic nerves, is unaffected by carboxypeptidase (124). However, not all the vascular effects of kinins are associated with vasodilatation, because in man and/or animals, kinins are reported to evoke constriction of pulmonary artery (see Guth, 66), venoconstriction in the rabbit ear (66), and contraction of arteriovenous anastomoses of the finger (72).

CONDITIONS EXHIBITING INFLAMMATION

The main vascular responses to kinins correspond to prominent features of the inflammatory response, viz., vasodilatation, increased vascular permeability, the local accumulation of leukocytes, and pain (125). Furthermore, the probable activation of Hageman factor and other pre-kininogenases by contact with injured tissues, and the release of cathepsins by damaged cells, would amply explain the formation of kinins in inflammation. On the other hand, the difficulty in demonstrating kinins in inflammatory exudates might well be due to their rapid hydrolysis by kininases (22).

Experimental injury.—Increased levels of bradykinin have been reported in blister fluid, serum and urine from patients with burns (126). In addition, Rocha e Silva (127) has accumulated considerable evidence that the edema evoked in the rat's paw by heating at 44–45°C for 30 min is accompanied by the release of kinin, the edema formation being strongly suppressed by cyproheptadine, imipramine, dibenzyline, and reserpine, and rather less so by chlorpromazine and dibenamine. But besides suppressing the effects of bradykinin, these antagonists also inhibit those of histamine, 5-HT, epinephrine, and acetylcholine. The intraperitoneal administration of cellulose sulfate or carrageenin (successive doses at 12-hourly intervals of 20, 20, 10, 10 mg/kg), respectively, depletes the plasma kininogen level by about 40 and 10 percent (128). Thermally induced edema is moderately suppressed in animals depleted in this manner, but the depletion regimen is probably not specific for kininogen. Accordingly, the overall results do not conclusively support the role proposed for kinins in the later stages of thermal injury (cf Rocha e Silva, 127).

The edema induced by injecting kaolin is claimed to be suppressed by large doses of soya bean or pancreatic trypsin inhibitor, but not by ovomucoid (129). Such results correspond to the ability of these inhibitors to antagonize plasma kallikrein, but similar suppression with these drugs has not been obtained in thermal or ultraviolet injury (130, 131).

In crush injury of rat's skin, the vascular permeability response has an intensity and time-course which correspond to the severity and duration of crushing (132). The early part of the response is decreased by antagonists of histamine and/or 5-HT, whereas the late effects are moderately suppressed by antagonists of kinin (pyridinolcarbamate) and 5-HT (132). In the same species, pyridinolcarbamate moderately suppresses delayed exudation in turpentine pleurisy and thermal burns of the skin (133); but, in addition to kinins (134) and kallikrein (133), pyridinolcarbamate also suppresses the permeability effects of lymph node permeability factor and RNA (135). As with thermal injury, therefore, there is at least some evidence that kinins are involved in the later stages of the permeability response in crush and chemical injury, although the various authors themselves admit that their evidence is inconclusive. However, the proposals gain some support from recent work in my department (136) which suggests that vascular endothelium contains two types of receptors (137); the first is activated by histamine or bradykinin, with stimulation being succeeded by refractoriness which lasts some hours. The second type of receptor is activated only by bradykinin, but in this case, refractoriness is induced by neither bradykinin nor histamine (136). These results therefore make it feasible that kinins might be responsible for prolonged permeability responses in inflammation, but as yet there is no conclusive evidence to support this possibility.

Pancreatitis.—The proteolytic and kinin-forming activities of trypsin, together with its ability to convert pre-kallikrein to kallikrein, provide grounds for suspecting that kinin formation may be a key factor in the pathogenesis of acute pancreatitis. The administration of a pancreatic inhibitor (Trasylol), which inhibits the effects of both trypsin and kallikrein, has been claimed to have therapeutic benefits (138), but a recent clinical trial (139) seemed to indicate convincingly that Trasylol had no value, except possibly in very large doses which approached toxic levels.

Arthritis.—Kinins have been demonstrated in effusion of the knee joint in gout (140), while the injection of bradykinin into the knee joint has effects which simulate arthritis and are aborted by carboxypeptidase B. In two gouty patients (140), an acute attack of arthritis evoked an increase of local bradykinin, whether the attack was spontaneous or induced by injecting micro-crystalline sodium urate into the knee joint. Since both Hageman factor (141) and PF/dil are activated by contact with the surface of glass or urate crystals, the above results have been suggested as evidence for a role of kinins in acute gouty arthritis. Attractive as this proposal might seem, however, it has been challenged on the grounds that the development of acute arthritis provoked by intra-articular injection of urate or calcium pyrophosphate is related to the local accumulation of neutrophil leukocytes (142). Whether the inflammation is provoked by lysosomes released from emigrated leukocytes (143) poses yet another issue, and one for which a conclusive answer would seem to require that the test animals are entirely, not partly, depleted of circulating neutrophil leukocytes.

To return to the reports of kinin-formation in arthritis, kinin levels exceeding those in gout have been described in synovial effusions from rheumatoid arthritis (45, 140, 144) and even from a case of psoriasis (140). The formation of kinins in rheumatoid arthritis has been postulated to result from activation of the kinin-forming system by antigen-antibody complexes (104), or by a combination of rheumatoid factor with denatured IgG and plasma (145). Once again, however, kinins seem to occur in increased amount in practically every circumstance in which they have been sought.

Granulomatous inflammation.—This condition is associated with foreign body reactions, as well as with hypersensitivity. The activation of Hageman factor by contact with substances such as silica and similar substances (which frequently cause foreign body reactions), or by antigen-antibody complexes (e.g., in granulomatous inflammation with an immunological background), has therefore been suggested as evidence for the importance of kinins in the pathogenesis of granulomatous inflammation. Kinin production certainly seems to be increased in these conditions (see Kellermeyer, 98), but again its significance remains to be established.

Kinins and granulocytes.—When preparations of normal leukocytes containing 60–80 percent granulocytes are mixed with plasma in vitro, the concentration of kinin is quickly increased two- to threefold (105). The generation of kinin is proportional to the concentration of granulocytes and demonstrable in 5 min, although kinin activity disappears in the next one to two hr (105). Earlier studies had demonstrated that neutrophils collected from inflammatory exudates in rabbits also promoted the formation of kinins, with kinin-forming activity occurring in both lysosomal and extra-lysosomal fractions of the neutrophils. On the other hand, kininase activity was confined to lysosomal fractions and differed from plasma kininase in substrate activity (146, 147).

The overall results have therefore been suggested to indicate that the activation of Hageman factor by injured tissue results in the release of kinin, which in turn is chemotactic for granulocytes. These cells then release more kinin and so accelerate the tempo of the inflammatory response. When the accumulated granulocytes begin to disintegrate, destruction of kinin exceeds its generation and the inflammatory response therefore subsides (105).

NONINFLAMMATORY EDEMA

Hereditary angioneurotic edema.—This is a rare familial disease, inherited as an autosomal dominant trait, in which recurrent attacks of acute noninflammatory edema involve the skin, subcutaneous tissues, underlying muscle, and mucous membranes of the pharynx, larynx, and gastrointestinal tract (148). When the respiratory tract is involved, the edema may prove fatal (149). Such patients lack a serum inhibitor (150, 151) which antagonizes both PF/dil and kallikrein, as well as the esterolytic activity of the first component of complement (C′1) (152, 153). The levels of serum kininogen are decreased shortly after a bout of edema (154), whereas during the attacks there is an increased level of C′1 esterase and a corresponding decrease of C′2 and C′4 (152, 155). Between attacks, the plasma from such cases yields a permeability factor whose generation is blocked by soya bean trypsin inhibitor (156). Since C′1 esterase is insusceptible to soya bean inhibitor, a possible interpretation of the findings is that C′1 esterase, perhaps through generation of C′2, activates plasma kallikrein and thereby causes depletion of plasma kininogen (37).

VARIOUS OTHER DISEASES

The full list of diseases in which kinin formation is definitely, or possibly, increased includes various other conditions which fall into three broad categories: disorders associated with the vascular system, microbial infection, and hypersensitivity. The conditions include myocardial infarction, pulmonary edema, subarachnoid hemorrhage, migraine, intestinal obstruction, bacterial infection, trypanosomiasis, anaphylaxis, asthma, and the Shwartzman reaction (see Erdos, 157). Further elaboration of the findings would seem to serve little purpose, however, until the role of kinins in human disease has been assessed along the lines indicated below.

EVALUATION OF SIGNIFICANCE OF KININS IN HUMAN DISEASE

Kinins are, without doubt, formed in pharmacologically significant amounts in a wide range of diseases. How then can the significance of these polypeptides be assessed? The criteria for such evaluation fall into two categories (8, 158): (*a*) those supporting the plausibility of participation; and (*b*) those proving actual pharmacological mediation.

Criteria supporting plausibility.—The criteria which support plausibility include the distribution of kinins in appropriate tissues, their availability in active or activable amounts, their ability and potency to induce relevant pharmacological or pathological responses, and the presence of their natural antagonists or hydrolytic enzymes. However, these criteria provide no more than plausible evidence—otherwise we are likely to end up with conclusions like that which was deduced from the observation that 5-HT occurred in increased amounts in the carcinoid syndrome. The inter-relation of kinins with catechol amines, 5-HT and histamine, is undoubtedly complex (Fig. 7), but evidence from criteria such as the above does no more than provide a warranty to seek proof of actual mediation.

As indicated above, the case for kinins having a significant role in human disease rests on two main premises: 1. the outstanding pharmacological activity of kinins, particularly to induce vasodilatation, increase vascular permeability, evoke local tissue leukocytosis, and cause pain; and 2. their occurrence in significant amounts in conditions that exhibit prominent vasodilatation and hypotension, or the phenomena of inflammation. The case for kinins as vasodilators is mainly based on the work of Hilton & Lewis (159) concerning blood flow in the salivary gland following stimulation of the chorda tympani. Vasodilatation is considered to result from the secretion into the interstitial tissue of kallikrein, which then converts kininogen into kinin. Excess kinin not inactivated by local peptidases is probably eliminated via the lymphatics, together with unused kinin (22). However, this theory has been challenged by Schachter (160) on the grounds that the close-arterial injection of saliva into a gland with natural circulation results in vasodilatation that differs from that occurring when the chorda is stimulated in that increased blood flow after injection is slower in onset, less in extent, and more prolonged in duration. Furthermore, the vasodilatation quickly decreases in degree with repeated injections of saliva, although not with repeated stimulation of the chorda or injection of acetylcholine. This and other evidence (161) against kallikrein being the mediator in parasympathetic vasodilatation is supported by further evidence from Webster (124). The infusion of bradykinin directly into the salivary gland is accompanied by vasodilatation which is suppressed by carboxypeptidase B in the perfusate, whereas the vasodilatation in chorda stimulation is unaffected by carboxypeptidase (60).

I have indicated some of the arguments against the role of kinins as vasodilators simply to emphasize that this issue remains undecided. Furthermore, while opinion on this subject continues to differ between authorities such as Hilton & Lewis on the one hand, and Schachter and Webster on the other, there seems little justification for the expenditure of further effort (and money) on trying to identify kinins as mediators of clinical vasodilatation and hypotension simply by seeking to establish a temporal association of kinin with vasodilatation.

When we turn to the role of kinins as pharmacological mediators of the inflammatory response, the evidence is even less convincing. The inflammatory response is a complex event in which the individual vascular events themselves are phasic and probably result from different mechanisms (162). Vasodilatation and increased permeability may be dissociated during inflammation (131, 163, 164), while the same is true for tissue leukocytosis and the permeability response (165, 166).

Apart from the induction of vasodilatation in inflammation, kinins have also been proposed as important mediators of the main permeability responses that characterize injury of moderate or strong intensity (see 158, 167, 168). In support of this proposal, electron microscopic studies have revealed that the permeability effects of bradykinin are associated with the presence of inter-endothelial gaps in the affected venules (168, 169), just as with permeability factors such as histamine and 5-HT. However, the evidence for kinins playing a major role in late or delayed permeability responses (168) is mainly based on (*a*) studies of thermal injury (127, 128) which have already been discussed; and (*b*) the observation that the effects of histamine and 5-hydroxyptamine are probably confined to immediate and transient permeability responses (130, 170). Accordingly, the kinins have been proposed as principal mediators of the late and prolonged permeability effects in inflammation.

Criteria proving mediation.—The methods of identifying kinins as mediators of pathological events in human disease fall into two categories: 1. isolation of kinins; 2. suppression of their effects (*a*) by inhibitors of kallikreins or kinins, (*b*) by kinin analogues, or (*c*) by depletion of kininogen.

Isolation: The distribution of proteolytic enzymes in various tissues makes it likely that kinins would be produced under a wide range of circumstances. As a minimum for a meaningful association, therefore, the peak level of kinin should correspond to the maximal intensity of the corresponding response. Furthermore, for sustained responses, kinin must demonstrably continue to be produced in appropriate amounts. Nevertheless, it must be remembered that the identification of kinins may be hampered by the rapidity of their hydrolysis by kininases. In this context, kininase antagonists such as cysteine (171), thioglycollic acid (172), and various chelating agents (173–175) have been used to enhance and prolong the activity of kinins in vivo. But such potentiation of kinin-like activity does not represent actual isolation of relevant peptides.

Inhibition: Kallikreins are susceptible to a wide range of protease inhibitors of animal and plant origin (49). Such inhibitors include Trasylol, and at least two antagonists in plasma (176); of the latter, one is quickacting and the other slow in effect. The kallikrein inhibitors in plasma seem distinct from those for trypsin and chymotrypsin (177), but at this stage, our knowledge of these proteolytic antagonists remains unsatisfactory. Still other antagonists have been reviewed by Vogel, Trautschold & Werle (49).

The kinins themselves are inactivated by various kininases (see above), but the available antagonists of kinins have effects (127) which unfortunately are too nonspecific to give reliable results.

Analogues: Studies of the structure of bradykinin indicate that the arginines at either terminal (Fig. 2) have equal importance for biological activity (178, 179). Substitution of the phenylalanines in positions 5 and 8 usually produces analogues which retain some activity, whereas the three prolines seem to have varying importance. The glycine and serine residues probably serve as "spacers" to provide appropriate separation of the important functional groups (179).

Among a substantial number of analogues which have been synthesized, only a few have inhibitory properties (179). The inhibition of bradykinin is specific in tests using the rat uterus in vitro, but such inhibition is usually reversed when the dose is increased about 1000-fold. The in vivo value of analogues is therefore limited by this reversibility, as well as by the variation in inhibition for various tissues and by the instability of the inhibitory effects of these analogues when stored. Nevertheless, this instability on storage suggests that inhibition by these analogues might be due to an unstable derivative whose identification may permit the synthesis of useful antimetabolites (179).

Depletion: The association of kininogens with $\alpha 2$-globulins in plasma, tissue fluid and lymph, means that kininogen depletion in vivo is not feasible —at least, not until the various kininogens have been fully characterized.

CONCLUDING REMARKS

I appreciate that my comments on the role of kinins in various conditions may appear biased toward the dismissal of the polypeptides as important pharmacological mediators in human disease. However, I have no doubt that the enthusiastic protagonists of kinins will not be deterred by my few remarks in this comparatively short paper. My exhibition of bias is intended to highlight the flimsy foundation on which the kinin empire has been built, and to make a plea that less effort be devoted to trying to establish a role for kinins simply from their association with various diseases, and more effort to extending current studies of the substrates and formation of kinins, as well as the activation and antagonism of kininogenases and kininases. A conclusive assessment of the role of kinins in human disease does not appear possible until considerably more such information has become available.

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ANTIGENIC REVERSION IN HUMAN CANCER

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Studies based on the rejection of transplantable tumors between highly inbred or syngeneic rodents have established the existence of tumor-specific antigens in many animal tumors induced by either oncogenic viruses or chemical carcinogens (1–6). Normal tissue isografts exchanged between syngeneic animals contain identical transplantation antigens and survive indefinitely. The immunologic rejection of a transplanted tumor can therefore be evoked only by new, tumor-specific transplantation antigens (TSTA) which have arisen in the tumor tissue during the process of malignant transformation.

Both ethical restraints and the virtual absence of a genetically homogeneous human population have precluded the use of tumor transplantation as a tool in the antigenic analysis of human tumors. Alternative experimental approaches have, therefore, been necessary in human cancer immunology (7). In recent years, improved immunologic methodology and an adequate consideration of the necessary controls have permitted the demonstration of tumor-specific antigens in certain human cancers (6, 8–12). In the present review, however, consideration will be restricted to those tumors whose specific antigens apparently arise through a mechanism whereby the cancer cell reverts to the synthesis of constituents normally found only in fetal and embryonic tissues.

CARCINOEMBRYONIC ANTIGEN OF THE HUMAN DIGESTIVE SYSTEM

The term, carcinoembryonic antigen (CEA), was introduced in 1965 to designate a constituent found in all adenocarcinomas of the human digestive system, but which is normally present only in embryonic and fetal digestive tissues in the first two trimesters of gestation (13). The initial demonstration of the CEA followed a series of experiments involving adenocarcinoma of the human colon (14). This lesion was specifically chosen for study because its growth pattern is such that it does not extend intramurally for more than 6–7 cm either proximal or distal to the site of the visible tumor in the gross. Mucosa taken from surgical specimens beyond these points was, therefore, available as normal control tissue from the same donors who supplied the cancer material. One of the most perplexing problems in the antigenic analysis of human cancers, that of distinguishing tumor-specific from individual-specific antigenic differences, was thereby circumvented.

Antitumor antisera were prepared in rabbits and rendered tumor-specific either by absorption with an excess of corresponding normal tissue extract, or by utilizing the phenomenon of acquired immunologic tolerance. The antisera prepared by both procedures were tested for their content of antibodies by a number of different serologic procedures.

The results of these (14), and subsequent (15, 16), investigations revealed that all of the colonic adenocarcinomas examined contained an identical, qualitatively tumor-specific antigen which was absent from the corresponding, autologous normal colonic tissue. Appropriate studies excluded the possibility that the antibodies giving rise to these tumor-specific reactions were directed either against the bacterial flora of the bowel or the usually high concentrations of fibrin frequently found in malignant tumors.

By employing the tumor-specific system of the colon as a model, it was then demonstrated that all human adenocarcinomas arising from the entodermally-derived digestive system epithelium (esophagus, stomach, small bowel, colon, rectum, pancreas and liver) contain the same tumor-specific constituent. Although this component could not be detected in any other normal, diseased, or neoplastic tissues, it was found in embryonic and fetal gut, pancreas, and liver during the first two trimesters of gestation. For this reason, the material was named the carcinoembryonic antigen (CEA) of the human digestive system (13).

The CEA is a relatively large, water-soluble protein-polysaccharide molecule with a sedimentation coefficient of 7S-8S and a mobility in the β-globulin range upon immunoelectrophoresis in agar gel. The antigen has been purified by extraction in perchloric acid and the techniques of preparative chromatography and electrophoresis. Amino acid and carbohydrate analyses of such purified material have revealed a relatively consistent pattern from one digestive system tumor specimen to another (17).

Agglutination studies with tissue-cultured cells of colon cancer origin suggested that the CEA was a constituent of the tumor cell surface (18). This observation was confirmed by immunofluorescence microscopy employing frozen sections of digestive system tumors and fetal intestines (18, 19), as well as viable cells explanted from freshly resected colon cancers. In addition, a number of different specimens of viable colonic cancer tissue were incubated with a ferritin-anti-CEA conjugate and studied by electron microscopy for localization of the ferritin label. In this manner it was found that the CEA is situated, for the most part, in the glycocalyx of the tumor cell rather than the underlying trilaminar plasma membrane (20).

Tissues derived from a colon cancer and a liver metastasis of a gastric carcinoma had been maintained in organ culture for periods of seven and eight years, respectively. These tissues were recently examined for the presence of CEA by the immunofluorescence technique. Production of the antigen had persisted, albeit in small quantities, in both of these specimens (21).

In order to assess the natural immunogenicity of the CEA, the bis-diazotized benzidine hemagglutination technique was utilized in an attempt to demonstrate the presence of circulating anti-CEA antibodies in human sera. A specific, humoral anti-CEA antibody response was detected in approximately 70 percent of patients with localized digestive system cancer, and in a comparable proportion of pregnant women in all trimesters of pregnancy and the immediate postpartum period (22). At present, no function can be attributed to such antibodies in either of these two groups of individuals. In particular, the humoral anti-CEA moieties do not appear to be beneficial to the cancer-bearing host.

The fact that the anti-CEA antibodies fail to provide any apparent protection against tumor growth is not particularly surprising since the available evidence suggests that cell-mediated immunity, rather than a humoral immune response, is more likely to play such a role in both man and animals (23). A number of attempts have, therefore, been made to detect specific cell-mediated immune reactivity in patients bearing digestive system cancers.

Skin reactions of the delayed hypersensitivity type were observed in 17 of 19 patients with carcinomas of the colon and rectum when they were challenged, intradermally, with certain soluble membrane fractions obtained from autologous tumor cells (24). Negative reactions were observed when comparable normal tissue fractions were employed. The skin-reactive antigen was, however, also found in the digestive tract cells of both first- and second-trimester fetuses. Moreover, the CEA was detected in many of the preparations producing positive skin tests. Although they were found in the same membrane fractions, it still remains possible that the skin-reactive antigen may well be distinct from the CEA.

In vitro correlates of cell-mediated immunity have also been sought in patients with colon cancer. The colony-inhibition technique was used to demonstrate that the peripheral lymphocytes from such patients possess cytocidal properties directed not only against their own tumor cells but also against those obtained from colon cancers of other patients (25). Whether the CEA is the factor responsible for the cross-reactivity observed remains to be determined. It is, however, noteworthy that the incubation of peripheral blood lymphocytes from patients with digestive system cancer in the presence of CEA failed to stimulate a significant degree of lymphocyte transformation, as measured by the rate of ^{3}H-thymidine incorporation into DNA (26).

The presence of anti-CEA antibody activity in the sera of a majority of patients with localized digestive system cancers has already been considered. However, in those cases where the tumor had undergone metastatic dissemination, no such serum reactivity was detected at any time (22). Although the ease with which antibody molecules may leave the tumor capillary bed remains uncertain, it is possible that the surface-associated CEA might serve as a readily accessible "antibody sink." The presence of a large mass of tumor material could then lead to the adsorption and removal of the anti-CEA antibodies from the circulation.

A second possible explanation for the disappearance of demonstrable antibody activity with tumor spread is that the cancer tissue may release CEA directly into the circulation. With a large tumor mass, the corresponding serum concentration of the antigenic material would be relatively high, and might lead to CEA-anti-CEA complexes in antigen excess. The anti-CEA antibody constituents would, under these circumstances, no longer be available for participation in subsequent serologic reactions. In order to assess this possibility, a radioimmunoassay for the detection of circulating CEA was developed. The procedure employs standardized preparations of ^{125}I-CEA and heterologous anti-CEA antiserum, and is based on the principle of coprecipitation-inhibition in half-saturated ammonium sulfate. The sensitivity of the assay is such that concentrations of approximately 1–2 nanograms of CEA/ml of serum can be detected (*27*).

In the initial double-blind study of 200 patients, circulating CEA was detected in 35 of 36 patients harboring tumor tissue of either colonic or rectal origin. In keeping with the possibility that antigen-antibody complexes may form in the range of antigen excess in patients with metastatic tumors, it was found that such individuals do, in fact, have relatively high levels of circulating CEA. Of greater clinical significance, however, was the finding that substantial concentrations of serum antigen may be detected in patients with localized tumors of the digestive system. Furthermore, in a number of instances, detection of circulating CEA has been the first evidence of digestive system cancer and has preceded the demonstration of tumor growth by conventional radiologic procedures (*28*).

The presence of detectable levels of serum CEA is virtually diagnostic of digestive system cancer. Total removal of the tumor tissue results in a relatively rapid disappearance of the CEA from the circulation. Any residual tumor is, therefore, indicated by persistence of circulating antigen. In those cases in which the concentration of the CEA falls to an undetectable level following operation, the reappearance of antigen in the serum after several months would suggest a recurrence of tumor growth. Hence, this technique holds considerable promise as both a diagnostic and prognostic procedure in patients with digestive system cancer. The ultimate role of the radioimmunoassay for CEA in clinical medicine awaits more extensive application and a wider experience.

EVIDENCE FOR ANTIGENIC REVERSION IN OTHER HUMAN CANCER SYSTEMS

Indications that there might be antigenic similarities between human embryonic and tumor tissues have appeared intermittently during the last forty years. In many cases, the interpretation of results has been difficult because of the lack of adequate control studies (29–31). In recent years, the deficiencies in experimental design have been largely overcome.

Alpha$_1$-fetoprotein in human tumors.—Since 1944 (32) a variety of "fetoproteins" have been described in several mammals including humans (33, 34). These species-specific constituents are distinguished by the fact that they are present in fetal serum, but absent from the circulation of corresponding adult animals.

In 1963 (35) it was reported that certain chemically induced mouse hepatomas synthesized an α-globulin lacking in normal adult mouse organs, but which was antigenically identical to that found in embryonic and neonatal mouse serum. Just two years later, human α_1-fetoprotein was detected in the sera of patients harboring primary hepatomas (36). This observation has been repeatedly confirmed during the last five years (37, 38).

The frequency with which α_1-fetoprotein reappears in the sera of patients with primary hepatomas varies between 50–80 percent, and shows marked geographical variations. The incidence of fetoprotein-secreting hepatomas is higher in Africa than in Western Europe. There is, however, a good deal of variation between different parts of Africa (39).

Noncancerous diseases of the human liver apparently are not associated with the elaboration of α_1-fetoprotein. It was, however, recently demonstrated that 5 of 10 pyridoxine-deficient baboons initiated the synthesis of this type of material, although there was no indication that these animals had developed hepatocellular carcinomas (40). The possibility exists that hepatic pyridoxine deficiency is a premalignant state in which α_1-fetoprotein synthesis precedes the distinct morphologic evidence of tumor formation. On the other hand, it may be that the secretion of this material is associated with certain noncancerous diseases of the liver which remain to be recognized by more sensitive techniques.

At present the only nonhepatic neoplasm in humans which has been shown to produce α_1-fetoprotein is testicular teratablastoma. This phenomenon has been observed in about 10 percent of those cases in which it has been sought (39). Under these circumstances, the fetoprotein is synthesized by the tumor itself and not by the liver. The basis for this heterotopic synthesis of protein remains obscure. It is, however, no more surprising than the "inappropriate" secretion of protein hormones by a number of human malignant tumors (41).

Alpha$_2$H-ferroprotein in the sera of children with tumors.—The α_2H-globulin is another protein of hepatic origin which is normally present exclusively in human fetal organs and serum. It is not found in the sera of normal children beyond the age of two months. Nevertheless, this ferritin-like protein has recently been detected, by the technique of radioimmunodiffusion, in the sera of children suffering from a variety of cancerous conditions. Among the tumors which have been described in association with the elaboration of this material are nephroblastoma, neuroblastoma, teratoma, hepatoma, lymphosarcoma, reticulum-cell sarcoma, osteogenic sarcoma, and cerebral tumors. In all, 81 percent of the sera of 334 children with malignant tumors gave positive results. On the other hand, only 8 percent of 122 sera from children with noncancerous diseases were positive (42).

Placental alkaline phosphatase.—An alkaline phosphatase isoenzyme, normally absent from adult human tissues, has been identified in the sera of 4–5 percent of patients with various malignant tumors (43). The isoenzyme, which has been named for the patient in whom it was first detected (Regan), is biochemically and immunologically indistinguishable from placental alkaline phosphatase. This substance appears to be a product of the tumor itself, and has been extracted from human cancer tissue.

In those cases where the isoenzyme has been found, it has proved clinically useful in monitoring the progression or regression of tumor growth. Since the enzyme continues to be elaborated from the seedings of serosal surfaces, it may serve in identifying malignant effusions. Moreover, the detection of an unexplained but persistently elevated level of serum alkaline phosphatase in any patient should stimulate a search for an occult cancerous lesion.

Fetal sulfoglycoprotein antigen.—A fetal type of sulfoglycoprotein antigen (FSA) has been demonstrated, by double immunodiffusion in microplates, in the gastric juice of 75 of 78 patients who had histologically verified gastric cancers (44). The same material was, however, found in 14 of 148 individuals who were either normal or who suffered from noncancerous gastric pathology. A relationship of the FSA to carcinogenesis was suggested because secretion of the antigen apparently preceded the development of morphologically distinct cancer cells. Although this pathogenetic association remains to be more clearly defined, the detection of the FSA may prove of diagnostic value.

Heterophile fetal antigen.—It was recently reported that 8 of 1030 sera from patients with various types of cancers were found to contain an antibody constituent capable of precipitating saline-soluble extracts of 82 of 115 malignant solid tumors, including those of the breast, colon, ovary, kidney, parotid gland, muscle, bone, and nerve. In addition to the tissue content of the antigen, this material was found in 17 of 200 sera from patients with solid tumors or leukemias (45).

Although serum specimens from normal subjects and patients with noncancerous diseases did not contain the antibody, the antigen under consideration was found in 12 of 16 benign tumors of the uterus, breast, and thyroid, and in 2 of 55 specimens of non-neoplastic tissue. Unlike the fetal antigens of human cancer tissue previously considered, this substance showed no species-specificity and was found in the sera of human, bovine, porcine, canine, and feline fetuses. This new fetoprotein, which remains to be further defined, has an electrophoretic mobility in the mid-γ-globulin region (46), and is serologically distinct from both α_1-fetoprotein, and the CEA of the human digestive system.

THE MECHANISM OF ANTIGENIC REVERSION

On the basis of the foregoing discussion, it would appear that during the embryonic and fetal processes of differentiation, specialization, and organization which take place during the major part of human gestation, the developing tissues contain specific components which disappear toward the later stages of gestation and normally do not appear in adult life. However, during the anaplastic changes of disorganization (disorientation) and dedifferentiation (despecialization) associated with the process of malignant transformation, these components reappear as tumor-specific antigens absent from comparable normal adult tissues. The hypothetical mechanisms associated with the phenomena of the deletion of embryonic antigens from adult cells (47, 48) and the reappearance of these constituents in cancerous tissues may be considered briefly.

In the very early stages of human embryonic cellular division, each primitive cell is endowed with the totality of inherited genetic information. This genetic potential, which is passed on to all subsequent generations of somatic cells (49), could theoretically allow each cellular unit to specialize into any human cell type. As anatomical orientation is initiated under the influence of certain "organizers," distinct organ systems begin to develop. At this stage, the pluripotential cells prepare for the events which will ultimately lead to the formation of the adult structures. During this process, genetic repressor mechanisms, presumably comparable to those so elegantly demonstrated in bacterial systems (50), act first to suppress the areas of the nuclear genome not required for the differentiation of any given cell under consideration. However, those genetic potentialities necessary to allow that cell to undergo its particular type of specialization continue to be expressed. Upon adequate differentiation of a tissue prior to birth, certain of the cellular components needed during the course of specialization appear to be no longer required. At this stage, then, further repression of genetic information occurs and the production of these "primitive" components ceases.

During the events of carcinogenesis, a process which has been termed derepressive-dedifferentiation (13) or retrodifferentiation (34), leads to the expression of genetic information which is normally repressed in the mature cell which has not undergone malignant transformation. This, in turn, leads to the recommencement of cytoplasmic production of the fetal or embryonic components. It is presumably these constituents which may then be detected as the tumor-specific antigens, absent from the corresponding normal adult tissue.

At present, the function of these primitive components in the human cancer cell remains entirely unknown. It seems most likely that they result from, rather than stimulate, the process of malignant transformation. Nevertheless, the functions which these materials may have in maintaining the viability of the tumor cell in its dedifferentiated or retrodifferentiated state should not be minimized. In any event, the fact that these materials are unique to the tumor tissue, in the adult at least, has provided the oncologist with a highly desirable tumor-specific property which may prove invaluable in the diagnosis, and possibly even in the immunotherapy and immunoprophylaxis, of human cancer.

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SENILE MACULAR DEGENERATION[1]

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Macular degeneration is a nonspecific term encompassing diverse disorders. Most common is senile macular degeneration (SMD), a major cause of visual impairment in the elderly. In addition, there are several heredomacular degenerations which affect individuals in the first few decades of life (1), as well as cerebro-macular degenerations which rarely are limited to the macular region (2). Some of the latter may be due to "slow" virus infections, the best documented example being subacute sclerosing panencephalitis, which is caused by the measles virus (3). Maculopathies, both reversible and irreversible, secondary to medications such as the synthetic antimalarials (4, 5), topical epinephrine (6), and allopurinol (7) have recently been recorded. Degeneration of the macula may also occur in conjunction with other ocular disease. Wise & Wangvivat (8) have noted an exaggerated macular response in a number of retinal disorders. The most common feature of all forms of macular degeneration is some ophthalmoscopically visible derangement of the macular area. There are numerous clinical descriptions of the ophthalmoscopic appearance(s), often having associated eponymic terms, but rarely are such descriptions specific (9). Altered or diminished central visual acuity is the usual complaint of affected individuals.

This review will be concerned primarily with the topic of senile macular degeneration. Focus will be on newer findings dealing with this entity. In order for the subject to be meaningful a brief review of the regional anatomy of the macula is provided.

ANATOMY OF THE MACULAR REGION

The macula is the anatomic center of the retina and the region responsible for accurate form and color perception. In its center is the fovea which lies approximately 4.5 mm, or three disc diameters, lateral to and 0.8 mm below the center of the optic disc (see Fig. 1). The diameter of the macula is approximately 1.5 mm. In the freshly opened, enucleated eye the macula

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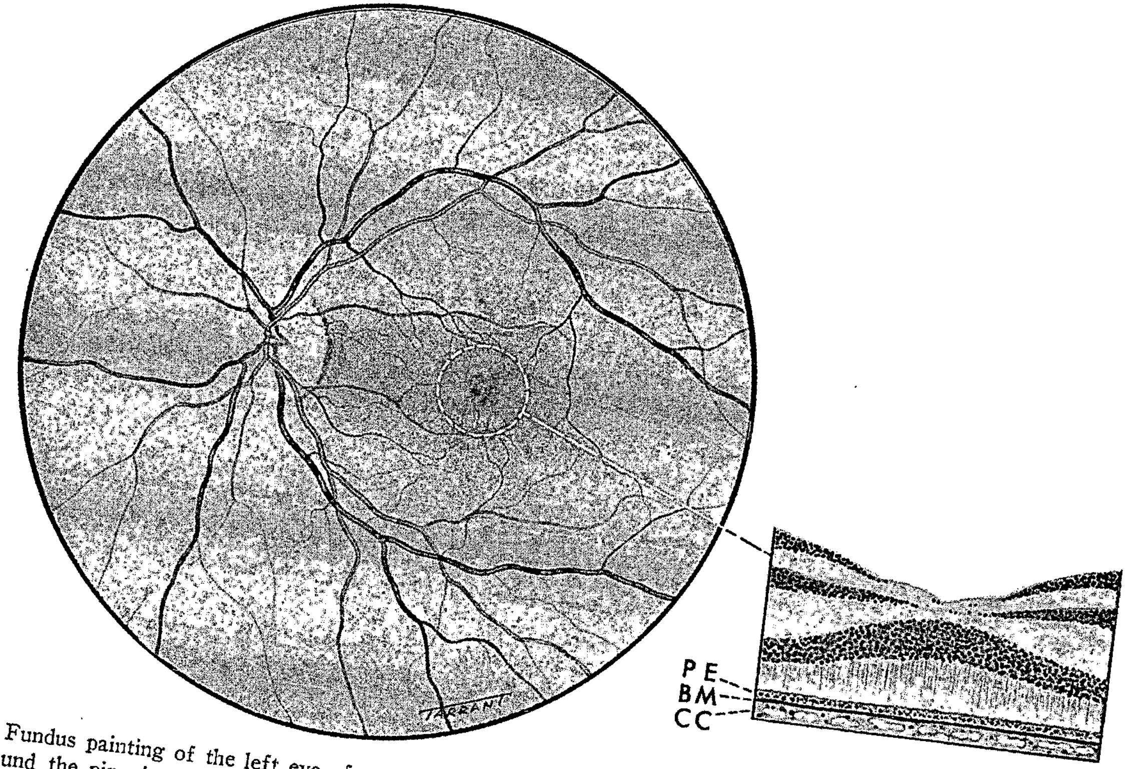

Fundus painting of the left eye of a human. The white circle outlines the macular region which is centered around the pinpoint fovea. Inset is a cross-section of the retina. The multi-layered retina, with its foveal pit lacking a ganglion cell and inner nuclear layer, lies upon the retinal pigment epithelium (PE). Bruch's membrane (BM) separates the choriocapillaris (CC) from the pigment epithelium. The outer layers of the choroid are not represented in this diagram.

has a yellowish color, hence the term macula lutea. This color is thought to be due to a xanthophyll compound. Two types of phoreceptors are present in the retina: rods and cones. The cones, which are the receptors responsible for photopic vision, that portion of vision characterized by the ability fo discriminate colors and fine detail, are particularly concentrated in the macula.

The retina has a very high rate of metabolism (10). Its nutritional needs are served by two separate sets of blood vessels: retinal and choroidal (11, 12). The inner or vitreal half of the retina, from the outer plexiform layer to the internal limiting membrane, is supplied by the retinal vessels. These vessels lack sympathetic innervation and have some properties in common with other vessels of the central nervous system. They have the ability to exclude certain substances, for example, fluorescein and particulate carbon, from entering the retina. This ability provides the so-called blood-retinal barrier (13). Choroidal capillaries nourish the outer half of the retina, mainly the photoreceptors and their nuclei. These capillaries comprise a layer of closely packed, broad vascular channels with thin, fenestrated walls. The entire capillary layer is termed choriocapillaris. The foveal region of the macula is indented and lacks the inner vascularized retinal layers, and for this reason it is thought that the major share of foveal nutrition comes from the underlying choriocapillaris. It has been pointed out that the short ciliary arteries, which bring blood to the posterior choroid, are concentrated in the macular region and that there may be a significant hemodynamic stress on the choroidal capillaries in this area (14).

Interposed between the regina and the choriocapillaris are the retinal pigment epithelium and Bruch's membrane. The former is a monolayer of cuboidal epithelial cells which contain a form of melanin. Recently, it has been demonstrated that the pigment epithelium has a phagocytic function, incorporating and digesting the shed outer segments of the rods (15). This property may be essential for proper rod photoreceptor function. No similar interaction of cones, which do not shed their outer segments, and the pigment epithelium has been demonstrated at this time. The retinal pigment epithelial cells are joined together by "tight" junctions (16) whose function may be similar to that of the zonulae linking the retinal vascular endothelial cells (17, 18). Indeed, both the blood-retinal and pigment epithelial-retinal barriers seem essential for maintaining a constant internal retinal milieu. The pigment epithelium is capable also of undergoing metaplasia and may form fibrous or osseous tissue.

Bruch's membrane is a multi-layered structure consisting of the basement membranes of the choriocapillaris and of the retinal pigment epithelium separated by irregularly arranged bundles of collagen and a core of elastic tissue (19). Though the function of this membrane is not certain, alterations in its structure appear to be significant in a number of conditions including (senile macular degeneration) SMD.

It is important to note that the sensory retina is only loosely adherent to the underlying retinal pigment epithelium, but that the pigment epithelium adheres firmly to Bruch's membrane. Thus, detachments of the pigment epithelium tend to be well circumscribed, whereas detachments of the sensory retina are usually more extensive.

INCIDENCE OF SENILE MACULAR DEGENERATION

The incidence of SMD is uncertain, but it has been reported to affect more than 25 percent of the aged population, particularly those over the age of 70 (20). Kornzweig (21), studying an institutionalized population of aged Jews, found macular lesions in 33 percent of 194 eyes which he examined clinically and histologically. On the other hand, it is unusual to find SMD listed as a major cause of legal blindness (generally considered to be vision of 20/200 or less in each eye) (22, 23). Many of the patients affected by SMD will accept the loss of central vision and not consider themselves blind particularly if their peripheral vision remains unimpaired. SMD is rarely reported from underdeveloped countries (24, 25).

CLINICAL PICTURE OF SENILE MACULAR DEGENERATION

There is a wide variation in the ophthalmoscopic appearance of SMD. The earliest change is often an easily overlooked pigment mottling of the macula. This may be accompanied by the appearance of multiple, irregular, yellow-white spots which lie deep to the retinal vessels. Some of these spots may be drusen, or hyaline deposits on Bruch's membrane, presumably derived from the pigment epithelium, but others appear to be deep-lying proteinaceous or fatty exudates. Vision is only minimally affected at this stage, and one can legitimately question whether the ophthalmoscopic appearance is a simple aging change or whether it reflects pathology. As the condition progresses pigmentary changes become more pronounced and they may be accompanied by the development of retinal and/or choroidal hemorrhages. The condition may remain stationary, with varying degrees of central visual loss, or progress to the state called senile disciform macular degeneration, or Kuhnt-Junius disease. This phase is characterized by an elevated macular lesion with subretinal exudate and hemorrhage. Neovascularization may be visible beneath the retina. Occasionally the macula becomes so elevated, and the subretinal contents appear so dark, that the diagnosis of choroidal malignant melanoma is entertained and the eye enucleated. In such cases, careful observation of the fellow eye may reveal some degree of alteration consistent with one or another phase of SMD, for the disease is usually bilateral if not symmetrical. It should be pointed out that not all authorities accept SMD and senile disciform macular degeneration as phases of one entity. Only recently have they been thought to represent variants of the same condition.

PATHOGENESIS OF SENILE MACULAR DEGENERATION

Though it is evident that there is eventual destruction of the photoreceptors in the macular region in SMD, the initial site of disturbance has been a matter of speculation. The larger choroidal vessels, choriocapillaris, Bruch's membrane, the retinal blood vessels, and the photoreceptors themselves have all been implicated in pathogenesis. Rarely, however, are eyes with the earliest stages of the condition available for histologic examination. Most reported specimens are either postmortem eyes lacking an accurate clinical history, or eyes with late disease. Recent advances in clinical examination, fluorescein angiography (26) in particular, and newer histopathologic techniques, including electron microscopy and retinal digestion (27), have shed some light on the pathogenesis of SMD.

It is unlikely that the retina per se is the initially involved tissue in SMD. Though it is conceivable that the cone photoreceptors could be selectively damaged by some unknown agent or process, such a phenomenon has never been demonstrated, nor would it explain the clinical picture. The electroretinogram (ERG), which is a recording of the electrical potential of the light-stimulated retina during dark or light adaptation, is usually normal in cases of SMD (28), but may be abnormal in heredo-macular degenerations. Recently a reduction of b-wave amplitude of the ERG during dark adaptation was found in patients with juvenile and senile macular degeneration, but the significance of this finding is unclear (29). It is doubtful that the nonvascular components of the retina, such as the various types of neuroglia, are primarily at fault in SMD. On the other hand, the role of the retinal vessels in the pathogenesis of the condition has been questioned. One author (30), examining fluorescein angiograms, has reported finding microaneurysms, capillary destruction, and neovascularization as the initial change in some cases of SMD. This has not, however, been our experience. Kornzweig and colleagues (31) have noted selective degeneration of perifoveal retinal vessels in digest preparations in cases of SMD. But this degeneration may be an aging change since similar alterations have been demonstrated in the peripheral retinal capillaries of aged individuals with presumably normal retinas (32). It is also of interest that fluorescein angiography reveals that the perifoveal retinal capillaries become dilated and abnormally permeable in a number of conditions including peripheral uveitis and retinal angiomas, but not in SMD.

By far the most likely site of initial pathology in SMD is the choriocapillary-Bruch's membrane-pigment epithelium complex. These three structures are so intimately related anatomically that they were once considered to comprise a single unit, the so-called tunica Ruyschiana.

Gass (33, 34), on the basis of histologic studies and fluorescein angiography, has suggested the following sequence of events for the pathogenesis of SMD: 1. degeneration and loss of integrity of the choriocapillaris; 2. degeneration and calcification of Bruch's membrane; 3. loss of adhesion of the pigment epithelium to Bruch's membrane and subpigment epithelial deposition of eosinophilic proteinaceous material and/or serous exudative detachment of the pigment epithelium and retina; 4. breaks in Bruch's membrane; 5. neovascular proliferation from the choroid through breaks in Bruch's membrane and into the subpigment epithelial space; 6. serous and/ or hemorrhagic detachment of the pigment epithelium and retina; 7. degradation, absorption and organization of the blood and exudate with proliferation, metaplasia and degeneration of the pigment epithelium; 8. secondary degeneration of the overlying retina. A number of authors (19, 35) deny that the choriocapillary bed is markedly involved in SMD, but there is little question that thickening of the intercapillary stroma around the choriocapillaris exists in many elderly individuals. Hogan (19), who has investigated the subject thoroughly favors Bruch's membrane as the initially involved tissue in SMD. He feels that degenerative alterations may occur primarily in the elastic and collagenous components of Bruch's membrane.

It is of interest that in one type of heredo-macular degeneration, vitelliform macular degeneration, an abnormality has been found in the electro-oculogram (EOG), which is a measure of the standing potential of the eye (36–38). It has been suggested that this EOG abnormality reflects a disorder of the retinal pigment epithelium. No similar alteration has been noted in SMD.

SMD appears to be a condition in which there is a great deal of overlap between physiologic aging and pathology. The question of why the macular region is involved selectively remains unanswered (39).

MANAGEMENT OF SENILE MACULAR DEGENERATION

It is apropos to quote Duke-Elder: (40) "No effective treatment is available." A wide variety of agents including vitamins, vasodilators, lipid-lowering agents, etc. have all been utilized without success. The slow course of the disease and the occasional regression of the exudative stage which permits some useful central vision must be considered when assessing the efficacy of medication. The successful closure of leaks in central serous retinopathy (a condition perhaps allied to SMD) by photocoagulation suggested this mode of treatment in SMD, particularly for the disciform variety. Early results were promising (41–43) but long-term follow-up has been disappointing. Low vision optical devices and large print books and periodicals have proven very helpful in allowing patients with SMD to keep visual contact with the world.

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OPTIC ATROPHY AND NEUROSYPHILIS 7007

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Two major recent advances in late syphilis of interest to every clinician have occurred in the past decade. These are: 1. the frequency of clinically progressive late seronegative syphilis; and 2. the presence of treponemes in patients with late syphilis despite penicillin therapy. The presence of treponemes in lymph nodes, despite penicillin therapy for syphilis, was first reported in both animals and man by Collart in 1962 (1–3). This observation was confirmed in rabbits by Del Carpio (4), and in man by Boncinelli (5). Of greater interest to the ophthalmologist was the discovery of treponemes in human aqueous humor, after administration of large doses of penicillin (6). This discovery was thereafter confirmed by Goldman (7), Jones (8), and Christman (9). Furthermore, study of a control group confirmed the absence of spirochetes in the aqueous humor of patients with senile cataracts, also examined by the same fluorescent antibody technique (10). Finally, the persistence of treponemes in the lymph nodes of both man and rabbit, despite treatment and even retreatment with penicillin, has been confirmed by the Venereal Disease Research Laboratory of the United States Government (11).

The importance of these observations is apparent. Two recent issues of *La Revue de Medecine (Paris)* were devoted exclusively to the subject of syphilis (12). Furthermore, the magnitude of the problem is evidenced by the fact that over 500 cases of late ocular and neurosyphilis have been detected on clinical grounds on the ophthalmological service of one city hospital in Miami, Florida, in the past eight years, and have been serologically confirmed by the FTA-ABS (fluorescent treponemal antibody absorption) test (13). For these reasons, several cases will be presented to illustrate important clinical, serologic, and laboratory aspects of late ocular and neurosyphilis. In the interests of space, primary emphasis will be given here to various forms of *optic nerve disease* encountered in late syphilis. These may be classified on the basis of ophthalmoscopic appearance (Table 1) or the associated visual field defects produced (Table 2).

CASE REPORTS

Case 1 (l.d.).—This 35-year-old, right-handed white female was seen July 19, 1966 through the courtesy of Dr. Feuerman. But for poorer vision in the left eye since childhood, she had no particular complaints until June 1966

TABLE 1. SYPHILITIC OPTIC NERVE DISEASE

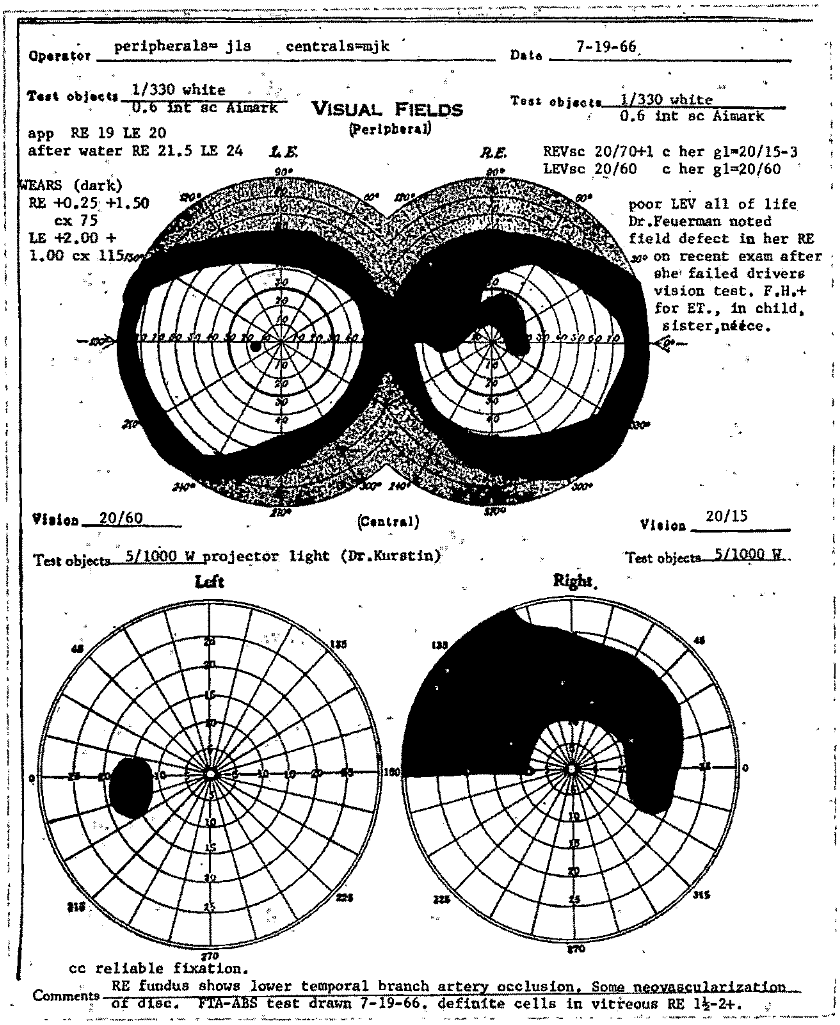
Ophthalmoscopic Classification
1. papillitis
2. primary optic atrophy
3. secondary optic atrophy
4. neuritis papulosa of Fuchs
5. perivascular sheathing
6. branch vessel occlusions
7. ischemic optic neuritis
8. segmental atrophy
9. consecutive optic atrophy

when she took the test for a driver's license and was advised to have an eye examination. A visual field defect was found in her right eye and she was referred for neuro-ophthalmologic evaluation. There were no other complaints. Past history revealed that she had been hospitalized five years previously at which time some fluid had been drained from her left hip. The cause for this monoarticular arthritis was undetermined at the time. The family history was positive for strabismus.

Examination revealed the corrected visual acuity to be 20/15 in the right eye and 20/60 in the left eye. The refractive error was significantly different in the two eyes and adequately accounted for moderate suppression amblyopia of the left eye. The external eye examination was normal. Slit lamp biomicroscopy disclosed a modest number of cells in the vitreous of the right eye. Visual field examination revealed an upper nasal nerve fiber bundle defect in the right eye (Fig. 1). Ophthalmoscopy disclosed a normal fundus on the left, but the right disc showed early neovascularization, and a lower temporal branch arterial occlusion was seen, with adjacent perivascular and bone corpuscular pigment distribution. The clinical impression was lower temporal branch artery occlusion in the right eye due to ocular syphilis.

TABLE 2. SYPHILITIC OPTIC NERVE DISEASE

Perimetric Classification
1. concentric contraction to red targets
2. concentric contraction to white targets
3. irregular peripheral wedge defects
4. nerve fiber bundle defects
5. central scotomas
6. caecocentral scotomas
7. altitudinal defects
8. ring scotomas



Operator peripherals= jls centrals=mjk Date 7-19-66

Test objects 1/330 white / 0.6 int sc Aimark

VISUAL FIELDS (Peripheral)

Test objects 1/330 white / 0.6 int sc Aimark

app RE 19 LE 20
after water RE 21.5 LE 24

REVsc 20/70+1 c her gl=20/15-3
LEVsc 20/60 c her gl=20/60

WEARS (dark)
RE +0.25 +1.50
cx 75
LE +2.00 +
1.00 cx 115

poor LEV all of life
Dr.Feuerman noted
field defect in her RE
on recent exam after
she failed drivers
vision test. F.H.+
for ET., in child,
sister,néece.

Vision 20/60 (Central) Vision 20/15

Test objects 5/1000 W projector light (Dr.Kurstin)

Test objects 5/1000 W.

cc reliable fixation.

Comments RE fundus shows lower temporal branch artery occlusion. Some neovascularization of disc. FTA-ABS test drawn 7-19-66. definite cells in vitreous RE 1½-2+.

FIGURE 1. Visual fields showing upper nasal nerve fiber bundle defect in right eye (Case 1).

Serologic testing on 8–26–66 by the Florida State Board of Health revealed a nonreactive VDRL test but a reactive FTA-ABS test. This was repeated on 8–28–66 and the Department of Ophthalmology Laboratory confirmed a nonreactive VDRL test but a 2+ reactive serum FTA-ABS test. Lumbar puncture on 9–15–66 revealed a clear, colorless fluid, with 2 monocytes, protein 25 mg percent, nonreactive spinal fluid VDRL test, and

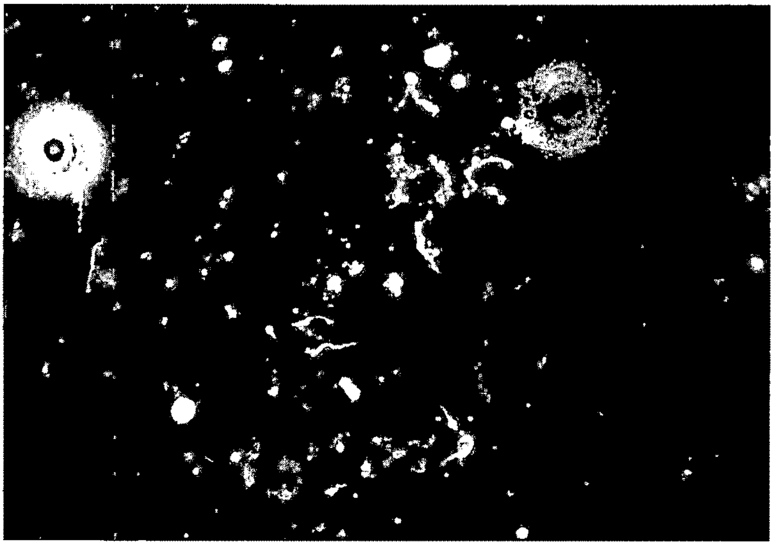


FIGURE 2. Oil immersion photomicrograph of lymph node biopsy cryostat FA section showing numerous *Treponema pallidum* organisms (Case 1).

a 0000000000 colloidal gold curve. The cerebrospinal fluid was centrifuged immediately after collection, however, and was darkfield-positive.

Aqueous humor paracentesis of the right eye was darkfield-negative, but positive with the fluorescent antibody (FA) stain for *Treponema pallidum.* An inguinal lymph node biopsy was done under local anesthesia, and numerous spirochetes were found in the node, which were motile on darkfield examination. These treponemes stained brilliantly with fluorescein-tagged anti-*T. pallidum* globulin on cryostat sections (see Figures 2 and 3). The patient was then treated with large doses of penicillin.

COMMENT

Routine eye examination revealed a nerve fiber bundle visual field defect in a thin normotensive young woman. The eyes were white and quiet but for a few cells in the vitreous and a branch artery occlusion in the fundus of the involved eye. Repeated serological tests revealed that the routine serum reagin (VDRL) test was nonreactive, but the FTA-ABS test was reactive. There was no history of previous syphilis obtained in this case. It was of interest that fluid had been aspirated from her left hip five years earlier, since the commonest cause of a monarticular inflammatory joint effusion is gonococcal arthritis. Be that as it may, this patient had a nonreactive serum VDRL test, but spirochetes were found in aqueous humor, cerebrospinal fluid, and inguinal lymph node, and were confirmed by both darkfield examination and the immunofluorescent stain for *Treponema pallidum.*

Case 2 (rjm 907409–1).—This 51-year-old, right-handed white man was first seen March 14, 1970, through the courtesy of Dr. Sime with a chief complaint of hazy vision for three months. About four-five months earlier he began to note difficulty differentiating red and green traffic lights, and blurred vision had gradually progressed thereafter. There were no other complaints. Past history revealed that he smoked two packages of cigarettes daily for twenty years and that he drank six beers per weekend. He admitted having received hip and arm shots for four months in 1943 for syphilis and stated that his blood test was "doubtful" ten years ago. Family history was negative for poor vision.

Eye examination revealed a corrected visual acuity of 20/200 in the right eye and 9/200 in the left eye. Visual fields disclosed bilateral caecocentral scotomas (Fig. 4). Ophthalmoscopy revealed definite bilateral primary optic atrophy. Neurological examination by Dr. David revealed that, despite intact vibration and position sense in feet and toes, the patient had reduced knee jerks, absent ankle jerks, and totally absent pain response at the Achilles tendons bilaterally.

The laboratory studies were of great interest in this patient. Skull and optic canal roentgenograms were negative and a brain scan was normal. Serological tests revealed a nonreactive VDRL but a reactive serum FTA-ABS test. Because of the reactive serum FTA-ABS test, a routine lumbar puncture was done. The cerebrospinal fluid on 3–18–70 was clear and colorless, but had 62 leukocytes/mm^3 which were 90 percent mononuclears and

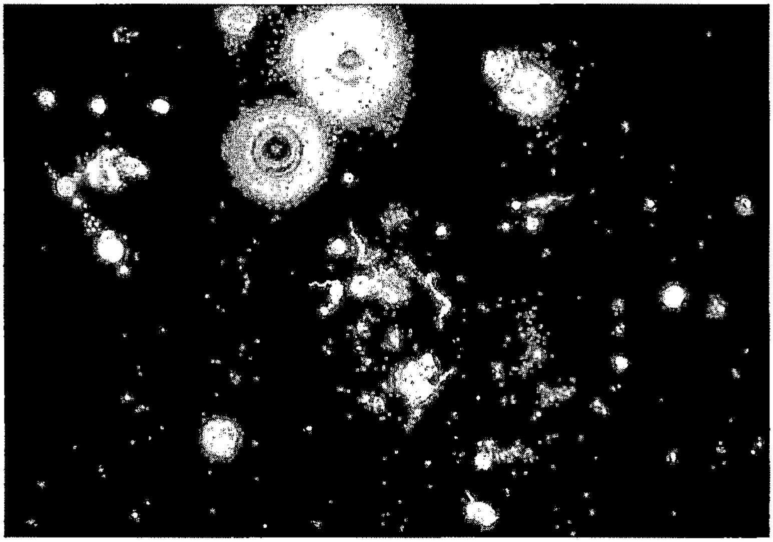
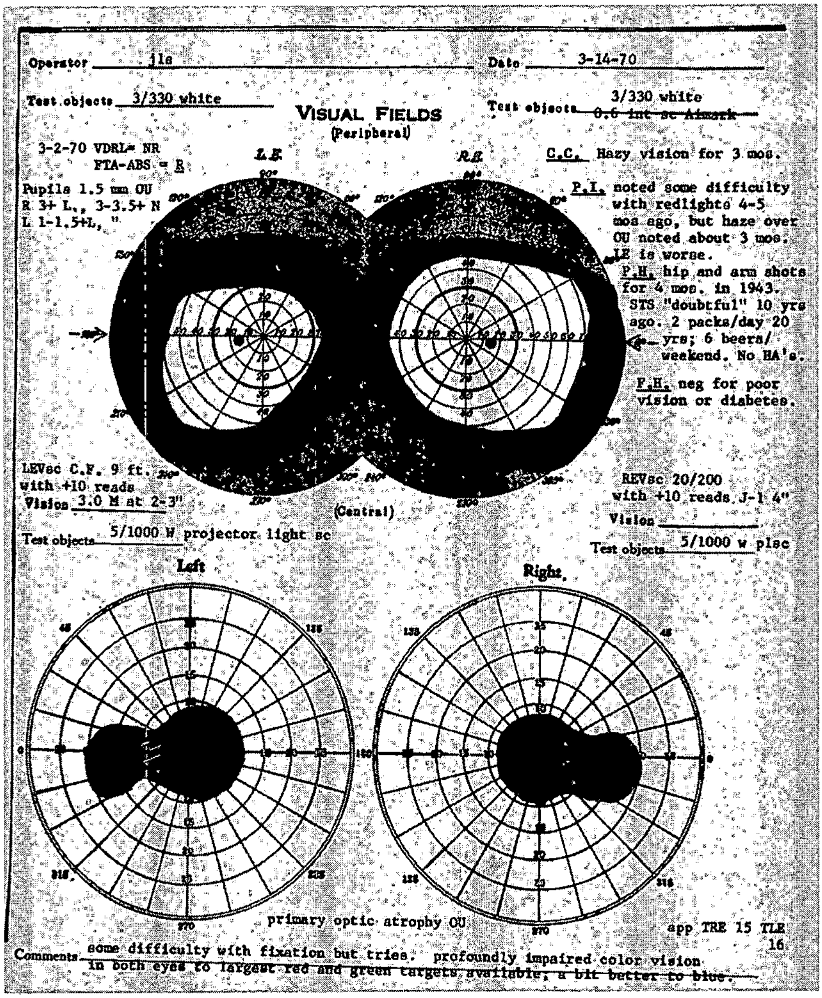


FIGURE 3. Treponemes in lymph node in Case 1.



Operator jls

Date 3-14-70

Test objects 3/330 white

VISUAL FIELDS
(Peripheral)

Test objects 3/330 white 0.6 int sc Aimark

3-2-70 VDRL= NR
FTA-ABS = R

Pupils 1.5 mm OU
R 3+ L., 3-3.5+ N
L 1-1.5+L, "

C.C. Hazy vision for 3 mos.

P.I. noted some difficulty with redlights 4-5 mos ago, but haze over OU noted about 3 mos. LE is worse.

P.H. hip and arm shots for 4 mos. in 1943. STS "doubtful" 10 yrs ago. 2 packs/day 20 yrs; 6 beers/ weekend. No HA's.

F.H. neg for poor vision or diabetes.

LEVsc C.F. 9 ft. with +10 reads
Vision 3.0 M at 2-3"

REVsc 20/200 with +10 reads J-1 4"
Vision

Test objects 5/1000 W projector light sc

Test objects 5/1000 w plsc

primary optic atrophy OU

app TRE 15 TLE 16

Comments some difficulty with fixation but tries. profoundly impaired color vision in both eyes to largest red and green targets available; a bit better to blue.

FIGURE 4. Visual fields showing bilateral caecocentral scotomas (Case 2).

10 percent polymorphonuclears, the total protein was 77 mg percent, and the spinal fluid VDRL test was reactive.

The clinical impression was syphilitic optic nerve disease, although the possibility of superimposed toxic amblyopia could not be excluded. Although early clinical signs of tabes were found, the patient had an essentially asymptomatic active neurosyphilis discovered by a routine lumbar puncture. He was advised to abstain from tobacco and alcohol, to eat a well-balanced diet, and was treated with penicillin and erythromycin. Three months later, the visual acuity was unchanged.

Comment

A 51-year-old man presented the typical clinical picture of toxic amblyopia with a history of moderately heavy tobacco and alcohol ingestion and bilateral caecocentral scotomas. Serum VDRL test was nonreactive but the FTA-ABS test was reactive. He gave a history of heavy metal therapy for syphilis 27 years earlier. Routine lumbar puncture revealed a mononuclear pleocytosis, elevated protein, and a reactive spinal fluid VDRL test. This unexpectedly active neurosyphilis was found in a patient with a nonreactive serum VDRL test, and again emphasizes the importance of the serum FTA-ABS test in the clinical diagnosis of late syphilis.

Case 3 (vf).—This 53-year-old, right-handed white woman was first seen March 17, 1969, through the courtesy of Dr. Pfeifer with a chief complaint of blurred vision and difficulty with memory. She had been well until one year earlier when the gradual onset of blurred vision began, followed by progressive somnolence, dysequilibrium, and mental confusion. Complete neurological examinations, serum reagin test, brain scan, and pneumoencephalography, done elsewhere were all reported as negative.

Eye examination revealed a corrected vision of 9/200 in the right eye and 7/200 in the left eye. A Marcus Gunn pupil was evident on the left. Dense bilateral central scotomas were noted and primary optic atrophy was present in both eyes, more marked on the left. The patient was pathologically somnolent and went to sleep once or twice during the examination, and tended to confabulate at times when reading a near card. Impression was primary optic atrophy associated with late seronegative syphilis, or with a diffuse abiotrophy of the nervous system, as corticopontocerebellar degeneration. The serum FTA-ABS test was nonreactive on 3–17–69.

After further careful study, the patient was advised by Dr. Reynolds to have an exploratory craniotomy to exclude a subfrontal meningioma. At surgery on March 28, 1969, no tumor was found but the arachnoid around the optic chiasm appeared thickened, and biopsies were made of both dura and arachnoid near the chiasm and submitted in the fresh state for examination. The dura was negative, but smears of arachnoid revealed two treponemes which were typical in morphology and which stained with fluorescein-tagged anti-*T. pallidum* globulin.

Repeat examination of the patient on April 4, 1970, revealed little change in the ensuing year since surgery. The vision was 8/200 in the right eye and 6/200 in the left eye, bilateral optic disc pallor was present, horizontal ocular dysmetria was noted, and there appeared to have been slight progression in the mental status changes. The patient was placed on ampicillin and benemid therapy.

Comment

A 53-year-old woman developed a progressive neurological illness characterized by blurred vision, somnolence, dysequilibrium, and confusion. Examination revealed primary optic atrophy, with bilateral central scotomas, some dementia, and ataxia. Surgical exploration of the optic chiasm revealed no evidence of a subfrontal tumor mass but the arachnoid near the chiasm was thickened and was biopsied. Despite nonreactive serum VDRL and FTA-ABS tests, a diagnosis of optochiasmatic arachnoiditis was established in this case, and to our knowledge this was the first instance of documenting treponemes in the human arachnoid in vivo with the fluorescent antibody stain for *T. pallidum.*

Case 4 (443544).—This 53-year-old, right-handed Negro man was first seen May 27, 1967, through the courtesy of Dr. Glaser for evaluation of "retinitis pigmentosa." The patient noted the onset of night blindness in 1960. In 1963, a pigmentary retinopathy was described in the Eye Clinic, and an electroretinogram was obtained which was reported as subnormal, and worse in the left eye. The patient had known hypertension and in 1965 suddenly developed a severe right-sided headache and incoherence, and was operated on April 24, 1965, with evacuation of a right temporal lobe intracerebral hematoma. He was left with a permanent left homonymous hemianopia following this event. The patient's vision was recorded in 1965 as 20/40 in the right eye and 20/100 in the left eye. Family history was negative for serious eye disease.

Examination on May 27, 1967, revealed a visual acuity of 20/30+1 in the right eye and light perception in the left eye. The right pupil was 2+ reactive to light and near, but the left pupil showed an amaurotic response. Ophthalmoscopy revealed typical retinitis pigmentosa-like fundi bilaterally. Further history was taken which revealed that the patient had gonorrhea 29 years earlier, and had taken injections for 10 days at that time.

Serological studies on this patient are seen below.

	VDRL	*FTA-ABS*
2- 4-65	NR	
4-26-65	NR	
4-11-67		+−NR
9-14-67	NR	+−NR

The patient was treated with 1,200,000 units of im Benzanthine penicillin G for 6 weeks between April–May 1967, for a total dose of 7.2 million units. Lumbar puncture was performed on September 23, 1967 which revealed a clear, colorless fluid, containing 1 monocyte, 3 erythrocytes, total protein 75 mg percent, 0000000000 colloidal gold curve, and a nonreactive spinal fluid VDRL test. However, examination of this fluid revealed spirochetes by both darkfield examination of the freshly spun sediment and with the fluorescent antibody stain for *T. pallidum* (see Figures 5–9).

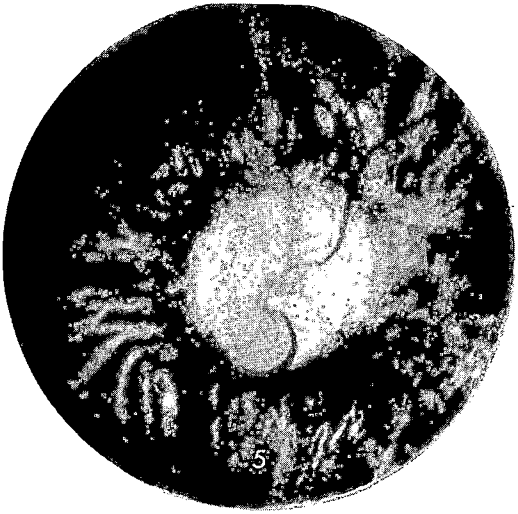


FIGURE 5. Fundus photograph of right eye of Case 4 showing optic atrophy.

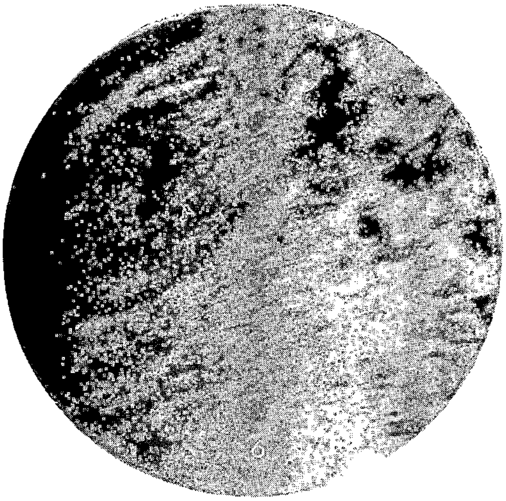
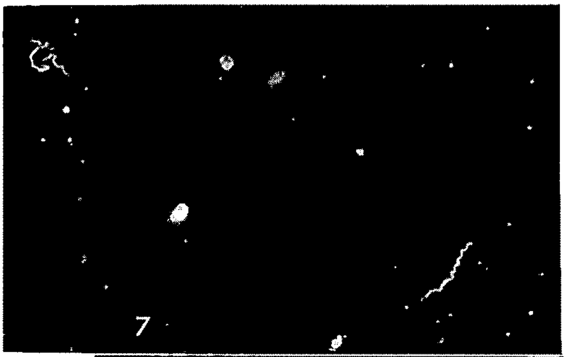
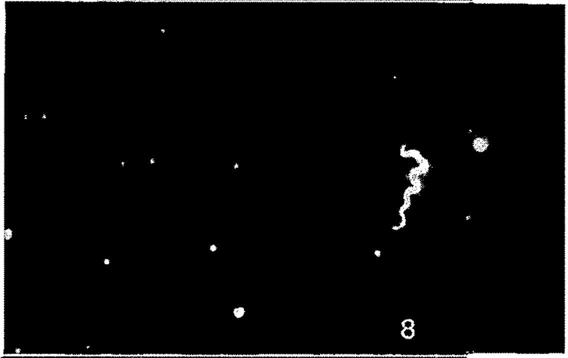


FIGURE 6. Fundus photograph of Case 4 showing retinitis pigmentosa-like change at equator.





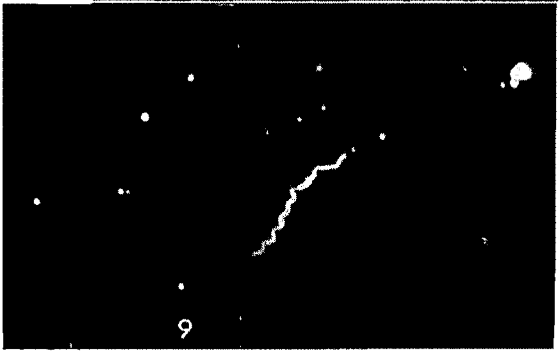


FIGURE 7. Cerebrospinal fluid of Case 4—tungsten illumination oil immersion photomicrograph showing spirochetes.

FIGURE 8. Spirochete in spinal fluid (Case 4) on FA smear.

FIGURE 9. Treponeme in spinal fluid (Case 4)—note perfect morphology. These organisms stained with fluorescein-tagged anti-*T. pallidum* globulin.

COMMENT

This patient was presented as an ophthalmoscopically classic case of true retinitis pigmentosa. Serum reagin tests were repeatedly nonreactive, and two FTA-ABS tests were equivocal. However, examination of the cerebrospinal fluid revealed treponemes of classic morphology which stained with the fluorescent antibody stain for *T. pallidum*. These organisms were found four months after the patient had received over seven million units of intramuscular penicillin therapy.

TABLE 3. Summary of Serological Studies on Case 5

Date	Blood			Cerebrospinal fluid
	VDRL	*RPCF*	*FTA-ABS*	*VDRL*
9- 4-68	NR			
11-18-68				NR
1-28-69	brain biopsy performed			
1-31-69	NR			
2- 2-69	R	R		
2-14-69		R		
2-19-69	started on procaine penicillin (24 million units over 10 days)			
2-20-69				NR
2-24-69	R	R		
3- 7-69			R	
4- 2-69	R	R		
4- 9-69				NR
4-23-69	R (4 dils)			

NR = nonreactive. R = reactive.

Case 5.—The following case is being reported elsewhere in detail by Ch'ien, Hathaway & Israel (14), but because of its importance is briefly cited here. A 38-year-old Caucasian male was seen at the University of Alabama Medical Center on November 13, 1968, complaining of frontal headaches for six months. There was a history of psychiatric illness for at least 15 years. This included inappropriate and grandiose behavior and attempted suicide. He gradually had become seclusive, paranoid, and demented, and was unable to recognize his brother by August 1968. With the onset of severe frontal headaches, hospitalization was advised. Past history was negative for venereal disease but he admitted having a urethral stricture.

Examination revealed a grossly abnormal mental status. He was disoriented, had a short attention span, and showed impaired judgment and recent memory. Otherwise, physical and neurological examinations were reported as within normal limits.

Detailed laboratory studies on this patient were normal and have been reported elsewhere (14). Serum VDRL tests were nonreactive on two occasions (see Table 3). Cerebrospinal fluid VDRL tests were nonreactive on three examinations (see Table 4). Pneumoencephalography revealed slight

TABLE 4. Pertinent Cerebrospinal Fluid Data in Case 5

Date	Cell Count	Protein	VDRL	Colloidal Gold
9- 9-68	3 polys	62		0011111000
11-18-68			NR	
12-16-68	0	70		0111000000
2-20-60	0	63	NR	1223210000
4- 9-69	0	66	NR	1233321000

dilatation of the ventricular system and slight widening of the sulci indicative of mild cerebral atrophy.

Because of difficulty in diagnosis, a brain biopsy was performed on January 28, 1969. Histopathologic examination at both the University of Alabama and the Armed Forces Institute of Pathology revealed the classic changes of active general paresis. The meninges were thickened and showed a diffuse infiltration by lymphocytes and plasma cells (Fig. 10). Perivascular cuffing was striking in the meninges, underlying cortex, and white matter (Fig. 11). There was a marked increase in astrocytes and microglial cells in the cortex (Fig. 12). A portion of the biopsy was submitted unfixed and frozen. Examination of this tissue was definitely positive with the indirect immunofluorescent stain for *Treponema pallidum.*

Comment

A 38-year-old man presented with a progressive psychiatric disease of 15

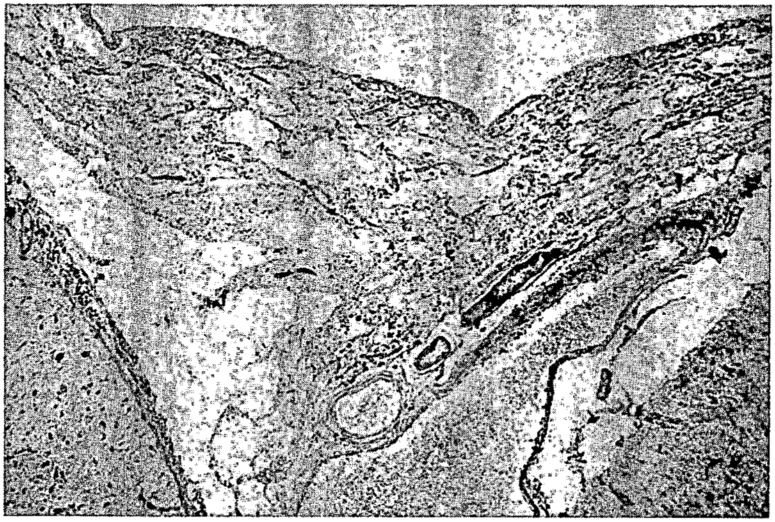


Figure 10. Hematoxylin and eosin photomicrograph (50×) of brain biopsy of Case 5 showing diffuse infiltration of meninges with lymphocytes and plasma cells.

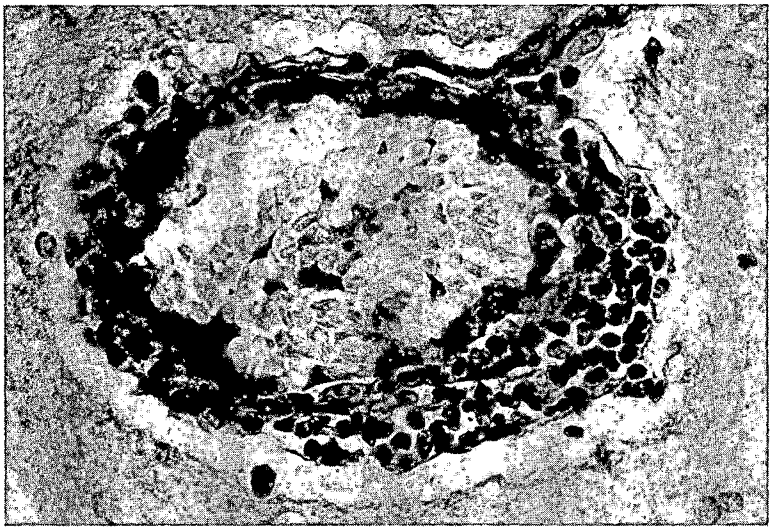


FIGURE 11. Perivascular cuffing in frontal lobe cortex of Case 5 (500×).

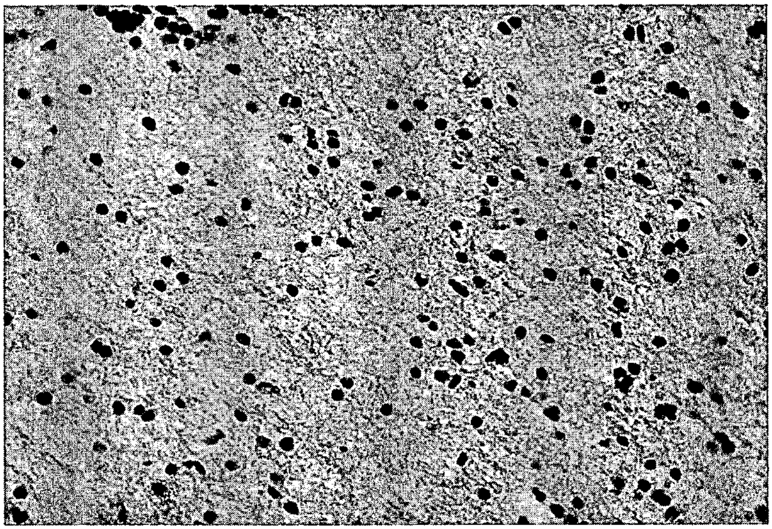


FIGURE 12. Hypercellularity of cortex of Case 5 (50×)—changes typical of active paresis.

years duration. VDRL tests were nonreactive on serum on two occasions, and on the cerebrospinal fluid on three occasions. However, brain biopsy established the histopathologic diagnosis of active general paresis. It is of interest that following surgery, seroconversion was demonstrated to the VDRL test. Furthermore, after the diagnosis had been histopathologically established, further serologic studies were performed. Both the RPCF (Reiter protein complement fixation) and the FTA-ABS tests were reactive. This case is the first in vivo demonstration of *Treponema pallidum* in the human brain by the fluorescent antibody technique. It is also an interesting example of active paresis in a patient seronegative to reagin tests.

DISCUSSION

Spirochetes have now been demonstrated repeatedly in patients with clinical signs of late syphilis irrespective of the serological findings and regardless of prior administration of penicillin therapy. Treponemes have been found in aqueous humor, cerebrospinal fluid, temporal artery, lymph node, liver, frontal lobe cortex, arachnoid, skin, and optic nerve of living patients, and at postmortem examination also in spinal cord, brain, and aorta as well. Treponemes found in aqueous humor and ocular tissue of a child treated with penicillin for congenital syphilis were found to be virulent *T. pallidum* on passive transfer to the experimental rabbit (15). The significance of these observations has yet to be fully appreciated by the profession.

Collart recently summarized (16) a review of immunity in syphilis as follows:

> From all the experimental facts expounded, we may conclude that immunity in syphilis should be considered as being very relative, since it is only applicable to one given strain (Kolle's monoimmunity), may be overcome by an inoculum which is more concentrated (Magnuson) or more virulent, and lastly, it depends on where (Chesney) and by which pathway inoculation takes place (McLeod). The most recent research results, now generally recognized, tend to show that this refractory condition, which may persist for some time after belatedly applied treatment, is not really immunity as Chesney understands it, but rather a modification of tissue receptivity which is maintained by the persistence of quiescent treponema in the body.
>
> As for recovery after early treatment, we have seen the experimental and clinical factors which enable us to consider this recovery in the sense of a bacteriological sterilization. This is no longer true if treatment is begun late. Indeed, all the works we have reported seem to indicate that we are dealing with a balance between, on the one hand, the host who has been sensitized by the infection which existed before therapy was begun, and who, because of this, has acquired a power of resistance, and on the other hand the parasite, vegetating in the body in the commensal state. But this does not exclude the possibility that under certain unpredictable biological conditions, unknown to us, the germs may recuperate all or a part of their virulence and become pathogenic once again, at least for the host who harbours them. Undoubtedly this latent state may appear clinically as a re-

> covery, since a considerable number of patients do not show, and will not show any manifestations throughout their entire lifetime. But this does certainly not correspond to "bacteriological sterilization."

A stronger statement than this might be made by a recent study of the prognosis of general paresis after penicillin treatment reported from the Epidemiology Branch of the National Institute of Neurological Diseases and Blindness (17). A follow-up study of 103 patients diagnosed as having general paresis on the active rolls of St. Elizabeth's Hospital in Washington, D.C. was made in January 1966. Follow-up of 100 patients with general paresis who had survived at least 10 years after initial treatment revealed that at least 31 percent had developed new signs of neurological disease subsequent to treatment. In fact, of patients who had received penicillin alone, 39 percent had developed new neurological signs. The authors stated that these rates are probably conservative because only gross signs such as convulsions or hemiparesis were accepted. They documented 14 cases of grand mal convulsions, and 17 cases of paraplegia, hemiparesis, amyotrophy, tabes, optic atrophy, and oculomotor palsy occurring after treatment. No attempt was made to evaluate progression of mental deterioration or worsening of neurological signs which existed before treatment in this study. Treatment included 3–30 mega units of penicillin. There was no correlation between size of dose and likelihood of developing new neurological symptoms. The authors further concluded that progression of the disease was common in the absence of a reactive spinal fluid and, conversely, that a reactive spinal fluid did not always presage clinical progression.

Finally, changes in clinical concepts of late syphilis continue to occur at a rapid rate. The recent demonstration of reactive serum FTA-ABS tests and atypical beaded fluorescence (18) in patients with disseminated lupus erythematosus should incite vigorous research efforts to determine whether or not treponemes are present in the aqueous humor and cerebrospinal fluid of such patients. Clinically, a disease producing lesions of skin, eyes, blood vessels, kidneys, liver, and central nervous system, with not infrequently reactive serological tests for syphilis, should not be lightly dismissed as a "biological false positive" reaction. An atypical immunologic response to infection with *Treponema pallidum* would be a logical explanation for the cause of lupus erythematosus.

Further confirmation of the presence of treponemes in the aqueous humor of patients with active uveitis, and their absence in a control group of patients with routine senile cataracts, has recently been reported, and again the documentation of organisms in the frequent absence of serologic reactivity was stressed (19).

In summary, the following points about late ocular and neurosyphilis should be emphasized, based on the cases here reported and on findings in the recent literature:

1. Clinically active late syphilis is a common disease and frequently is found

in patients who are seronegative to the commonly employed (reagin) serological tests.

2. The serum FTA-ABS test is indicated in the clinical evaluation of any patient showing progressive disease of the eyes and nervous system, not otherwise explained, and regardless of the serum VDRL test findings.
3. Spirochetes have been found in the absence of any clinical or laboratory evidences of inflammation.
4. Spirochetes have been demonstrated in patients despite treatment with large doses of penicillin therapy in late syphilis.
5. Progression of paresis after treatment has been documented in one-third of the cases followed for ten years.
6. *Treponema pallidum* has been demonstrated with the fluorescent antibody technique in vivo in the human brain in seronegative patients.
7. Histopathologically active general paresis has now been documented in vivo in the human brain despite nonreactive VDRL tests in both serum and cerebrospinal fluid.

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MYASTHENIA GRAVIS AND THE THYMUS[1] 7008

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Myasthenia gravis is a disease wherein a lesion of neuromuscular conduction produces characteristic symptoms of weakness and easy fatigability; especially affected are the facial, oculomotor, laryngeal, pharynageal, and respiratory muscles.

Myasthenia gravis has long been known to be associated with abnormality of the thymus (1), and thymectomy for myasthenia gravis has been practiced regularly since 1939 (2). Yet it is only recently that immunological and experimental pathological studies have provided a clue as to the relationship between the thymus and defective neuromuscular transmission (3–5) and thus a rationale for thymectomy in myasthenia gravis.

These recent studies suggest that the thymus is an endocrine gland which secretes a hormone, termed thymin, which depresses neuromuscular transmission (5). An autoimmune reaction in the thymus, autoimmune thymitis, is considered to cause hypersecretion of thymin, which in turn causes the partial neuromuscular block of myasthenia gravis (4–6). Thus, myasthenia gravis, like thyrotoxicosis, may represent a hormonal hypersecretion due to an autoimmune reaction in an endocrine gland (7); in keeping with this concept it could, therefore, be re-named "thymotoxicosis."

SIZE OF THE THYMUS

A thymoma is present in 10–15 percent of patients with myasthenia gravis (8) and, due to the tumor, the weight of the thymus is increased and there may be a large anterior mediastinal mass since some thymomas grow to a considerable size.

In the absence of a tumor, thymic size is normal in myasthenia gravis. Thus, the weight falls within the normal range for age (5, 9) and there is no radiological enlargement, even when a discriminating technique such as anterior mediastinography is used (5, 10). This is an important point since, for many years based on erroneous concepts of thymic size, it was considered that the nontumorous thymus in myasthenia gravis was "persistent," "hypertrophied," or "hyperplastic" (11). It is not. The human thymus does not disappear after puberty (5) and the thymus in myasthenia gravis falls within the normal wide range of weight for age (5, 9).

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[2] Career Scientist of the Health Research Council of the City of New York.

THYMITIS

Thymoma occurs in only 10–15 percent of cases of myasthenia gravis (8) but there are certain histological changes in the thymic medulla which occur both in the thymus adjacent to a thymoma in a patient with myasthenia gravis (9, 12, 13) and in the normal sized thymus found in other patients with myasthenia gravis. These changes include the presence, in the medulla, of numerous large lymphoid germinal centers (9, 13, 14), increased density of small lymphocytes (14), and the presence of plasma cells (15, 16).

These changes in the thymus have long been termed hyperplasia (8, 9, 14), by analogy with the use of this term to describe similar changes in the lymph node. Yet, the lymphoid tissues are not hyperplastic in myasthenia gravis (9) and, although the thymus does contain lymphocytes, it is essentially an epithelial organ with an inductive function in relation to these lymphocytes (5). Unlike the lymph nodes, germinal centers are either absent or few in number and small in the normal thymus, and plasma cells are also scarce (5, 15). Thus, it would seem more appropriate to consider these histopathological changes in thymus as analagous to similar changes in the thyroid in thyroiditis or the salivary glands in sialadenitis (4).

By this analogy the lesion of the thymus in myasthenia gravis is thymitis, both in the nontumorous thymus and the thymus adjacent to a thymoma.

AUTOIMMUNE THYMITIS

The serum of at least 30 percent of patients with myasthenia gravis contains an antibody, first detected by Strauss (17), which reacts with the striations of skeletal muscle and also with myoid cells in the thymic medulla (17–19). These reactive cells in the thymic medulla were initially termed "epithelial" cells (18) until their myoid nature was appreciated. Myoid cells have rounded heads and elongated tails which may show the actual structural features of striated muscle (20–23). Thus, the myoid antibody of myasthenia gravis, which does not react with the neuromuscular synapse (24), also represents a thymic autoantibody since it is reactive with thymic myoid cells which are normal components of the thymus.

This serological finding in myasthenia gravis is, therefore, consistent with an autoimmune reaction to thymus and, in conjunction with the histopathological evidence for thymitis (vide supra) leads to the suggestion that autoimmune thymitis is the basic thymic lesion in myasthenia gravis (4, 5).

ETIOLOGY OF AUTOIMMUNE THYMITIS

In patients with myasthenia gravis and thymoma, the disease seems correlated with the presence of thymitis in the adjacent thymus (9, 12, 13). Approximately one third of all thymoma patients have myoid antibody in the serum (25) and, of these, one half have myasthenia gravis, these being patients in whom thymitis with germinal centers tend to be found in the adjacent thymus. Thus, the thymoma appears to induce an autoimmune reaction to myoid antigen and initiates autoimmune thymitis, a process similar to the induction, by thyroid carcinoma, of thyroid autoimmunization and thyroiditis (26).

In patients with myasthenia gravis in whom there is no thymoma, there is a clinical and serological overlap with other autoimmune diseases (3, 5) but the initiating cause of the autoimmune thymitis remains unknown.

EXPERIMENTAL MYASTHENIA GRAVIS

Experimental studies have provided support to the concept, derived from human data, that autoimmune thymitis is the basic thymic lesion in myasthenia gravis.

Experimental autoimmune thymitis was induced in guinea pigs or rats by injecting them with saline extracts of thymus or striated muscle in Freund's complete adjuvant (6, 27–32). Since striated muscle has common antigens with thymic myoid cells (19, 27), this is also equivalent to immunization with thymic antigens. Two weeks after immunization, aggregations of small and medium lymphocytes were found in the central medulla, surrounding Hassall's corpuscles, and this finding was interpreted as thymitis. In randomized sections, thymitis was found to be significantly related to immunization with thymus or muscle. Experimental autoimmune thymitis has been produced using bovine tissue extracts and outbred guinea pigs (6, 28, 30), and by using homologous tissue extracts in Hartley guinea pigs (27, 32), inbred Lewis rats (29), and inbred Wistar rats (31). With heterologous immunization in guinea pigs, antibodies to the cytoplasm of thymic epithelial cells and lymphocytes were formed in thymus-immunized animals and antibodies to muscle striations and thymic myoid cells in muscle-immunized animals (6, 28, 30). These antibodies were weak or did not develop in Hartley guinea pigs after homologous immunization (27, 32). Outbred guinea pigs immunized with bovine tissues also developed delayed hypersensitivity to these extracts but these cutaneous reactions were not tissue-specific (28, 30).

These histological and immunological studies suggest that autoimmune thymitis can be produced experimentally. Vetters et al (33) were unable to produce experimental autoimmune thymitis but this may have been due to technical problems and no immunological studies were reported in this experiment.

Animals immunized with thymus or muscle in Freund's complete adjuvant were found by electromyography to have an abnormality of neuromuscular transmission and this abnormality was correlated with the presence of histological thymitis. The electromyographic points of similarity between the neuromuscular block of animals with experimental thymitis and that of patients with myasthenia gravis included (*a*) decreased amplitude of muscle action potential with a single supramaximal nerve stimulus (27, 29); (*b*) significant increase in this muscle action potential following administration of anticholinesterase drugs, neostigmine (6), or edrophonium chloride (Tensilon) (30); (*c*) a significant decline in successive muscle action potentials with supramaximal nerve stimulation at a rate of 50/sec (6, 27, 29–31); and (*d*) a more sustained response of the muscle action potential with nerve stimulation at 50/sec following administration of anticholinesterase drugs (6, 30, 31).

Further evidence that animals with experimental thymitis have a partial neuromuscular block was obtained by studying events at individual motor end plates with microelectrodes. In studies of muscle biopsies from patients with myasthenia gravis, a characteristic abnormality was found: a reduction in amplitude of miniature end plate potentials (mepps) with normal frequency of mepps and a normal resting membrane potential (34, 35). This abnormality persisted in vitro despite thorough washing of the preparation. Similarly, in diaphragm preparations from rats with experimental autoimmune thymitis, there was a decrease in mepp amplitude which persisted despite thorough washing of the preparation, and mepp frequency and resting membrane potentials were normal (29). The decrease in mepp amplitude was also demonstrated in vivo in rats with experimental autoimmune thymitis by using the intertransversarius muscles of the tail in anaesthetized rats (29).

Thus, animals with experimental autoimmune thymitis have an abnormality of neuromuscular transmission that, by the electromyographic and microphysiological criteria studied, appears to be similar to that of patients with myasthenia gravis.

The relationship between thymitis and the neuromuscular block was evaluated by several experimental approaches.

First, thymectomy prior to immunization prevented the development of neuromuscular block despite normal immune responses in adult thymectomized animals (6, 30). Secondly, thymectomy at two weeks in animals with partial neuromuscular block reduced the persistence of electromyographic abnormality from the usual 7–10 days to 3–4 days (31). Finally, transfer of a "thymitis" thymus within a millipore chamber to a normal recipient caused a neuromuscular block to develop in the recipient (Kalden, J. R. 1969. Personal communication).

These experiments all point to a humoral substance released in excess with thymitis as the cause of the partial neuromuscular block.

THYMIN

Studies of neuromuscular transmission in thymectomized or thymus-grafted inbred Lewis rats suggested that this substance is being secreted physiologically by the thymus (36). In thymectomized animals the amplitude of mepps was slightly but definitely increased while in the presence of increased amounts of thymus there was a definite depression of mepp amplitude (36).

Thus, these findings suggest that the normal thymus does secrete physiologically a substance which depresses neuromuscular transmission as measured by mepp amplitude. The name "thymin" was proposed for this substance since it would appear to be a thymic hormone affecting neuromuscular transmission.

There is a long and controversial literature regarding the effects of thymic extracts on neuromuscular transmission (reviewed in 5). Repeated intraperitoneal injections of thymus extract, but not of muscle or lymph node extract controls, produced a myositis and a partial neuromuscular block in the diaphragm of guinea pigs (37). This partial neuromuscular block was similar to that of myasthenia gravis in that it was improved by neostigmine and was sensitive to *d*-tubocurarine (37). Repeated subcutaneous injections of these thymus extracts also produce a partial neuromuscular block in rats, detectable by electromography, and this thymus-specific substance, which is presumed to be thymin, is currently being studied in attempts to isolate it and determine its structure (unpublished observations).

THYMECTOMY FOR MYASTHENIA GRAVIS

The recent evaluation of thymectomy for myasthenia gravis dates from Blalock's 1939 report of a successful thymectomy performed in a patient with myasthenia gravis and thymoma (2). Controversy surrounded the operation for some time as clinicians were hesitant to perform an operation of such magnitude but of an unknown rationale in patients with a neuromuscular disease. However, the results of large series of thymectomies for myasthenia gravis are now available and these patients do show a striking benefit, with 70–80 percent having either a complete remission or substantial improvement (38, 39). This is in keeping with the concept of myasthenia gravis as thymotoxicosis. An explanation for those cases not improving with thymectomy may lie in incomplete thymectomy, the frequent presence of ectopic thymus tissue in man (5), or the presence of irreversible structural neuromuscular lesions with long-standing disease (5). It was frequently noted in the evaluation of thymectomy for myasthenia gravis that the prospects for cure were more closely related to shortness of duration of symptoms than to the degree of severity of the disease (38, 40), that is, long-standing cases developed irreversible neuromuscular lesions.

Thus, early and complete thymectomy would appear to be the optimal treatment for myasthenia gravis both in the light of the theoretical consideration of myasthenia gravis as thymotoxicosis and the empirical results obtained in large thymectomy series, now totalling several thousand patients.

The technique of thymectomy has largely followed the sternal-split operation suggested by Blalock (2). More recently, several groups have advocated a revival of cervical thymectomy (41–43) which, prior to the thirties, was the standard technique for thymectomies (44, 45). In one small series of patients with myasthenia gravis, the results have been comparable with the sternal-split operation and offered the advantages of easier postoperative management and a better cosmetic result (43). Thymoma and a low tracheostomy scar were taken as contraindications to the cervical approach.

CONCLUSION

Although myasthenia gravis and thymic abnormality have long been known to co-exist, recent studies relate them more closely than ever. Current evidence suggests that:

1. The thymus is an endocrine gland, secreting a hormone termed thymin, which inhibits neuromuscular transmission.
2. Autoimmune thymitis is the basic thymic lesion in myasthenia gravis,

initiated in 10–15 percent of cases by a thymoma. Autoimmune thymitis results in hypersecretion of thymin or "thymotoxicosis."

3. Early and complete thymectomy is both a rational and proved treatment for myasthenia gravis.

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ALCOHOLIC MYOPATHY

7009

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INTRODUCTION

As is so often the case in clinical medicine, the recent widespread recognition of alcoholic myopathy as a clinical entity was preceded by many years by observations made by earlier clinicians. James Jackson stated in 1837 (1) that neurologic diseases could not explain all of the signs manifested by alcoholic patients; he believed their muscles, too, had to be abnormal. Magnus Huss then reported muscle weakness in alcoholic patients in 1849 (2), and clinical and postmortem changes were described in 1889 by Seimerling (3), and in 1896 by Gudden (4). These observations then were forgotten until Hed et al (5–8) described several alcoholic patients with an acute muscular syndrome characterized by muscle swelling and tenderness, gross myoglobinuria and sometimes by renal failure. In the ensuing years, a wide variety of muscular abnormalities have been recognized in alcoholism, but the mechanisms responsible for the muscle disease remain unknown. The state of our knowledge of this subject at the present time will be summarized in this paper.

CLASSIFICATION OF ALCOHOLIC MYOPATHY

Several clinical forms of myopathy, each with a different onset, severity, and course, can be found in alcoholic patients. These include:

I Subclinical myopathy (biochemical changes only)
II Acute alcoholic myopathy
  A. with muscle cramps
  B. with diffuse muscle weakness
  C. with rhabdomyolysis and myoglobinuria
III Chronic alcoholic myopathy

The evidence that a subclinical form of alcoholic myopathy exists rests solely on the demonstration of increased serum levels of enzymes normally considered to be of muscle cell origin. Of those most commonly measured, creatine phosphokinase (CPK) has received the most attention (9–13), but glutamic oxaloacetic transaminase (SGOT) and lactic dehydrogenase (LDH) also have been studied (13). The serum CPK is most often elevated in alcoholic patients just after they are admitted to the hospital following severe alcoholic debauch. The proportion of such values reported to be abnormal has ranged from 9 of 9 (11) to 36 of 59 (12) to 36 of 44 (13). The time after hospital admission that the sample is taken for CPK measurement is of importance; the level decreases steadily once the patient is hospitalized and usually reaches normal in five to seven days (9, 11). SGOT and LDH also may be elevated but changes in the levels of these enzymes are less striking and abnormalities are found less frequently (10, 12) than in the case of CPK.

An additional important laboratory abnormality found in asymptomatic alcoholic patients is diminished response of blood lactic acid to ischemic exercise (9, 12, 21). Of 30 patients studied by the author, 23 had diminished rise of blood lactic acid in response to one minute of vigorous exercise with a single upper arm cuff occluding arterial flow. Like the serum enzyme levels, the blood lactic acid response returned to normal within two weeks in those patients who were restudied.

Even though abnormal CPK values are found in alcoholic patients without muscular symptoms, i.e., "subclinical myopathy," these patients frequently give the history of periodic muscle cramps and commonly show previously unsuspected muscle tenderness on physical examination (12). The distinction, then, between "subclinical" and "mildly involved" is not a sharp one.

Clinically apparent acute alcoholic myopathy usually has an abrupt, dramatic onset characterized either by severe muscle cramps or by pain and swelling of muscle associated with gross myoglobinuria. Less frequently, the initial symptom is rapidly developing muscle weakness. That form of the disorder characterized by cramps usually follows close upon a recent severe episode of binge drinking. Muscle cramps involving the entire body of the muscle begin in one or both lower extremities, usually in the calves, and may then involve other muscles. Isolated severe contraction of the quadriceps and rectus abdominis muscles has been observed, and at times the rectus abdominis contraction may persist long enough to suggest the presence of an abdominal mass. The contracted muscle is markedly tender, but tenderness is less severe in other muscles and almost always is worse in the lower than in the upper extremities. Cramps occur at intervals which vary from every 15 minutes or so to several hours apart and last from 15–30 seconds. These symptoms usually subside within 24 hours of onset. If the patient abstains from alcohol, normal strength returns within one to two weeks.

The frequency of this form of the illness is difficult to estimate. There is only one report that specifically addresses itself to muscle cramps (14), and only two other case reports could be found in which they are mentioned (13, 15). But innumerable other workers have told the author of their own observations of similar patients and the clinical picture, therefore, cannot be rare.

The better known form of acute alcoholic myopathy is that associated with gross myoglobinuria. The symptoms are so abrupt in their onset and associated with such severe local manifestations that the condition has been confused with thrombophlebitis. Sudden pain, swelling, tenderness of muscles, and severe weakness occur in chronic alcoholic patients following severe binge drinking. The tenderness often is so exquisite that even touching the skin lightly is terribly painful, and the involved extremity may swell to twice its normal size. Localization in the legs is usual, but the arms also may be affected. Other complications of severe alcoholism also may be present, and the picture of a disheveled, acutely ill patient with any or all of such findings as jaundice, nystagmus, hepatosplenomegaly, spider angiomata, tremor, and severe painful swelling of one or more extremities is both striking and unmistakable. Recovery may be protracted and often is complicated by acute renal failure secondary to the myoglobinuria (vide infra). The swelling of muscles lasts for days; muscle weakness lasts for much longer, and may progress to chronic alcoholic myopathy, to be described below.

Myoglobinuria is characteristic of the laboratory examination in acute alcoholic myopathy. In the myopathy with cramps the myoglobin is present in the urine in such small amounts that it can be detected only by immunochemical means (13, 14, 16), but in patients with severe acute myopathy gross myoglobinuria is demonstrable by more usual methods (12, 17).

Other laboratory abnormalities are the same as those which are found in asymptomatic alcoholics admitted after severe binge drinking. Elevation of serum levels of CPK and other enzymes is almost uniformly present in acute myopathy (5–8, 12–15, 18, 19). Diminished response of blood lactic acid to ischemic exercise, too, is present in most cases in the author's experience (12), and has been found by others in the few instances in which the appropriate studies have been carried out (18).[1]

In addition to the patients with acute myopathy, chronic alcoholic myopathy now is recognized to be a common complication of severe alcoholism (6, 12, 13, 15, 18). The same clinical features are reported by each observer. All patients are severe chronic alcoholics, usually 30 years old or older. Complaints of muscular weakness are prominent in the history. While some patients have had previous episodes of acute alcoholic myopathy, the majority present with recent severe drinking, weakness, and diffuse muscle atrophy which is worse in proximal than in the distal muscles. The lower extremities are affected more often and to a greater extent than are the upper extremities, but the arms, shoulder girdle, and even the facial muscles occasionally are involved. Symptoms generally reflect the site of involvement. Difficulty arising from a sitting position and going up steps is common, while difficulty in raising the arms over the head or with facial movements is much less frequent. Most patients have diffuse muscle weakness underlying these more localized symptoms and may show muscle tenderness in addition. The tenderness, like the weakness, is more likely to be prominent in the calves and thighs than elsewhere.

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[1] Frawley, T., Nygren, A. Personal communications.

Associated abnormalities typical of the neurologic complications of chronic alcoholism also are frequent, occurring in half or more of the patients. Signs of peripheral neuropathy, confusional states, movement disorders or encephalopathy may be present and may complicate the clinical picture. The neurologic findings, however, do not explain either the proximal localization of the weakness or the muscle tenderness.

Like the acute myopathy, the prognosis of chronic myopathy is good if the patient stops drinking. All patients observed have improved by the time of discharge from the hospital, and complete recovery of muscle size, strength, and function occurred in some patients followed for a period of months while they abstained from alcohol. Other patients undergo striking improvement and then appear to stabilize with some residual and presumably long-lasting disability.

The laboratory findings in chronic myopathy are similar to those seen in the acute forms of the disease, but the abnormalities are less striking and are less frequent than in the acute myopathies. Serum enzyme levels may be elevated, particularly the CPK (6, 12, 13, 15), and diminished response of blood lactic acid to ischemic exercise sometimes also is found (12). Creatinuria sometimes is present (12).

Electromyographic studies have been made on many patients, but with variable results. The myogram may be normal (5, 6, 12, 15, 18) or show a variety of changes suggestive either of myopathy or of myopathy combined with neural lesions. Thus, potentials of short duration and low amplitude are found, as are diphasic and polyphasic forms (6, 15, 18). Fibrillations and reduced nerve conduction velocity also have been noted (12).

The histopathology of alcoholic myopathy varies greatly depending on the stage of the illness at the time the biopsy is performed. During the subclinical phase, when elevation of serum enzymes and decrease in response of blood lactic acid to ischemic exercise are the only abnormalities, minimal changes are noted (9). Modest nuclear rows and granular cytoplasmic change may be seen, together with occasional focal atrophy of muscle cells. During this stage, necrosis is unknown. In the acute myopathy with muscle cramps (22) light microscopy shows occasional random small fibers, sarcolemmal nuclear rows, some endymysial fat and granular cytoplasmic change. Isolated fibers containing clear vacuoles also are seen, but more dramatic changes are not noted. On the other hand, when acute myopathy occurs with gross myoglobinuria the changes are more striking, and necrosis, interstitial edema, and hyalinized and fragmented muscle fibers are found (5–8, 12, 20). Evidence of regeneration also may be present (20, 21). When sections are stained with toluidine blue, distinct areas of pale-staining, watery cytoplasm can be seen to alternate with zones of pale striations. Some fibers are found in which cross striations have disappeared. Individual fibers appear rounded and swollen, suggesting intracellular edema with increase in size of individual muscle cells.

Electron microscopy in the acute myopathy sometimes shows phagocytes (18, 19) but usually the changes are not those of inflammation (20). Increased cytoplasm is seen to separate the myofibrils, and the sarcolemma varies from intact, to minimally separated, to completely disrupted. The increased cytoplasmic space contains normal amounts of glycogen, ribosomes, and other organelles, but is filled with a finely reticular network of faintly stained filaments. The mitochondria sometimes are damaged, with blurred cristae (19), but usually are normal except for random dispersion in the distended cytoplasm. The myofibrils themselves show loss of the usual regularity between the A and I bands, and the width of the Z discs and the A and I bands varies markedly. Individual filaments of the I bands may be discontinuous and lucent areas frequently separate individual filaments. The pattern of the A band also may be disrupted.

In chronic myopathy the picture on light microscopy is complex. In addition to a mild increase in fibrous tissue (20), focal infiltration with fat is seen, sometimes with fat cells in a single line or in larger segments replacing destroyed fibers (6). Muscle fibers vary greatly in size, within a single fasicle from 5 to 50 millimicrons in diameter. Some fasicles may contain only small fibers, suggesting associated denervation atrophy. Focal replacement of cross striations with dark staining material sometimes is seen, and osmophilic globules occasionally are noted beneath the sarcolemma. Increased nuclear rows commonly are found (6, 15, 20). Electron microscopy often shows normal fibers, but small fibers with frequent nuclei and fat droplets are seen, and entire individual fibers and focal areas within other fibers may be found in which collections of clear cytoplasm interrupt or separate the fibrils. All that remains of a cell may be an empty basement membrane, and areas can be found in which only a few mitochondria survive; these may show dense inclusions in their matrices. The myofibrils themselves may be frayed and discontinuous, with the I band usually more severely involved than the A band or Z disc. However, focal involvement of the A band does occur, and manifests itself as irregular collections of fibrils in amorphous masses.

Regeneration also has been noted (20, 21) and replication of the basement membrane and separated cell processes containing abundant ribosomes, filaments and structures resembling Z discs are seen. These changes appear to indicate actively proliferating fibers. Thus, evidence of acute damage, chronic change, and regeneration all may be found in a single section.

These findings have led one group of workers to look carefully at autopsy material from patients with alcoholism not known to have myopathy (22). In contrast to the minimal findings by light microscopy of biopsies of alcoholics without muscular symptoms, the muscles of alcoholics who die in the hospital show a remarkable incidence of abnormalities. Histologic evidence of acute rhabdomyolysis was observed in 22 of 100 consecutive autopsies of male chronic alcoholics with fatty or cirrhotic livers. Both degenerative and regenerative changes were found. The changes, which included disruption of myofilaments, and shrunken mitochondria in the presence of numerous rhabdomyoblasts, were severe in eight patients and may have been associated with death from acute renal failure in two.

The relationship between alcoholic myopathy and alcoholic cardiomyopathy, liver disease and hematologic disorder is not clear. Liver disease and anemia may occur in patients with myopathy, and often do (12). On the other hand, while alcoholic cardiac disease has been reported in conjunction with the skeletal myopathy (23), the two seem to occur more often separately than together. This appears to be so even though there is no evidence that alcohol affects any tissue by a different mechanism from that by which it affects any other. This is an unsettled issue, however, and some have even proposed that the different skeletal muscular syndromes have different mechanisms (6). The clinical observation that the muscular disorders merge from one into the other suggests that this is not the case (12).

The pathogenesis of the disease is not known, and only a few laboratories have studied the possible mechanisms. Douglas et al believed the problem in acute myopathy was one of ischemia (19); there is no evidence to affirm or deny this hypothesis. Nutritional deficiency has been considered the underlying cause, as it has for all of the complications of alcoholism (9). The only effort to study this aspect of the problem experimentally is that of Dimberg et al (10) who provided suggestive but not conclusive data showing that alcoholics eating low carbohydrate diets had higher serum enzyme levels during periods of alcohol ingestion than did those receiving normal diets. This would not appear to be the entire story, however, since infusions of ethanol into normal subjects and alcoholic patients have given delayed rises in serum CPK (13), leading to the suggestion that a metabolite of alcohol might be the responsible offending agent (13). A variety of other studies also have been performed.[2] Measurements of CPK and serum potassium in 19 patients—and of serum magnesium in 25 alcoholic patients—have shown no correlation between CPK levels and decreased serum electrolytes even though hypokalemia and hypomagnesemia both are known to be associated with changes in muscle function and/or histology (24, 25). Short-term infusions of ethanol into dogs in amounts giving lethal blood ethanol levels have produced no changes in serum CPK, and mildly intoxicating blood levels of ethanol achieved during social drinking were not associated with significant change in CPK in 50 normal subjects studied.[2] Phosphorylase activity was normal in the muscle of patients with acute myopathy (9) and incubation of cell-free extracts of muscle of normal or alcoholic men, or of rats, in the presence of glycogen and large amounts of ethanol failed to inhibit lactate production.[2] Similar negative results ware obtained when acetaldehyde was included in the incubation medium in amounts comparable to those achieved in the blood of alcoholic patients during severe drinking bouts.[2]

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[2] Perkoff, G. T., Karl, I., and Counts, S. Unpublished observations.

More promising but still preliminary results have been obtained with rats in long-term ethanol feeding experiments. Pair-fed rats were adapted either to increasing amounts of ethanol up to 25 percent ethanol as their source of drinking water or to isocaloric amounts of 10 percent dextrose. Both groups were compared to those given ad lib chow. After nine months of pair-feeding, analyses were made of a wide variety of intracellular substrates and enzymes of muscle. Ethanol-fed animals showed distinct decreases in muscle lactate compared to their pair-fed controls and to chow-fed animals. They likewise showed increased citrate, malate, fumarate and isocitrate, but no change in ATP, ADP, AMP, or phosphocreatine. Glucose-fed animals compared to both alcohol and chow animals on the other hand, showed increases in glycogen, glucose 6-phosphate, alphaglycerol phosphate, 3-phosphoglycerate, pyruvate, and lactate.[3]

These results in rats suggest an effect of ethanol on intracellular muscle carbohydrate metabolism and are compatible with the observation of reduced blood lactate response to ischemic exercise in alcoholic patients. It is not known whether the changes found in rats bear directly on the clinical and anatomic changes seen in patients with alcoholic myopathy. The anatomical findings suggest an additional mechanism which either could result from an intracellular metabolic abnormality or be caused by a direct effect of alcohol on the cell itself. The wide separation of intracellular organelles by what appears to be increased cytoplasmic fluid, together with the known loss of intracellular enzymes and proteins into serum and urine (e.g., CPK and myoglobin), suggest that a major increase in cellular permeability is one of the earliest changes which occurs, and indeed might be the primary abnormality. How this might come about is as yet unclear.

This review must close, then, on an uncertain note. A wide variety of clinical disorders of muscle function do occur in alcoholism, and much more is known about their clinical, laboratory, and anatomical features than was the case a short time ago. But as is true with our understanding of the other complications of alcoholism, the pathogenesis of the myopathy is unknown. It seems clear that whether the mechanism involves nutritional deficiency, an effect of alcohol on carbohydrate metabolism or directly on the cell wall, a major increase in permeability of the cell to intra and extracellular constituents occurs that appears to be related directly to the clinical features of the illness. Fortunately, the treatment is clear, and if it can be adhered to, is effective. Abstinence results in significant improvement in alcoholic myopathy.

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[3] Perkoff, G. T., Karl, I. Unpublished observations.

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FETAL INDICATIONS FOR THERAPEUTIC ABORTION

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There are, of course, those who hold on grounds of personal opinion or individual morality that there are no fetal indications whatever for the early termination of a pregnancy. Their belief teaches that a "soul" enters the conceptus as the sperm penetrates the egg, and they use such phrases as "intrauterine murder" or "fetal euthanasia."

While all faiths have teachings which differ in some degree on this subject, the belief that a person is present in the uterus from the moment of conception is exclusively a teaching of contemporary Catholicism, and is not shared by Protestant, Jewish, Mohammedan, Buddhist, etc. For a person of Catholic persuasion, the management of congenital defects is to identify, classify, and institutionalize the damaged newborn but under no circumstances to prevent such a birth.

If such a belief were universal, there would be no need to write the present chapter, and the penalty for criminal abortion would be a life sentence for murder rather than a short term for felony. Despite shades of differences among other tenets, in general they hold that an individual is not present until individual existence can be maintained separate from the mother. Maternally dependent tissue on the uterine wall is living tissue, of course (as is the uterus itself) but it is not a person.

In such a view, the early termination of pregnancy is a legitimate therapeutic procedure with both maternal and fetal indications. In general, the medical profession has looked upon evidences of fetal defect as being the principal fetal indication for interruption and has indicated a preference for fetal death to defective life. Other factors (socio-economic status, the "wantedness" of a baby, etc.) have generally been grouped with the maternal indications.

In our experience at Johns Hopkins in the first two years after the liberalization of the Maryland law, fetal indications accounted for less than 2 percent of the total indications; with total repeal, this relative figure would be still lower since presumably the number of abortions for maternal indications would increase.

The American Law Institute's phraseology reads "substantial evidence of severe mental or physical defect," and has not, to the best of the writer's knowledge, been tested in court. Thus, for example, maternal diabetes is associated with a 9 percent chance of congenital defect, whereas the nondiabetic population carries a 3 percent chance of such an abnormality. Would the court hold that diabetes per se constituted "substantial" evidence of a fetal risk since it increases the possibility of congenital anomaly by 300 percent, or that it did not fit this definition since 91 percent of the progeny would be uninfluenced? This question has not been settled, although it would seem superficially unthinkable that a court would hold that 49 damaged babies out of every 100 pregnancies would not be a substantial risk simply because these babies were in the minority.

This example serves to indicate one of the bases on which fetal risk is determined,—viz. statistical correlation. In these circumstances the exact diagnosis of the state of a particular fetus cannot be, or is not determined with precision, but from known series of reported cases, or from Mendelian principles a general statistic is derived which gives the relative risk on a percentage basis. Armed with this figure which indicates the general magnitude of the gamble, the potential parents and the physician can discuss the wisest course to follow.

It should be pointed out in this connection that the important factor involved in such a gamble is not the odds, but the stakes. A 10 percent risk may not sound like very much, but a lifetime of a blind, deaf and mentally limited child constitutes a maximal penalty. The person who profits by "playing the odds" is the gambler who bets frequently enough that these odds have a chance of evening out. The average family of today, however, is going to take the gamble of reproduction from two to four times at most, and under such circumstances, being presented with the threat of a "15 percent risk" (for example) can be either misleading or useless as an aid in making a rational decision.

Two other comments must be made for completeness. The first is that while some ten states have liberalized their abortion laws in the past ten years, and 20 more may in the next decade, there is the distinct possibility that within the next two years all restrictive abortion laws may be eliminated by a Supreme Court decision declaring them unconstitutional. This will not alter the fact that there will be pregnancy terminations which are primarily carried out for fetal indications, but will mean that many physicians now comfortably hiding behind the law and ignoring this topic will need to clarify and render precise their own attitudes.

Secondly, any discussion of preganancy termination chiefly for fetal indications is carried out on the basic assumption that there is little maternal risk involved in the procedure. This is, in 1970, a valid assumption. Experience both here and abroad has indicated that in-hospital abortion is indeed a comparatively innocent procedure. While the earlier in pregnancy the operation can be carried out the safer, from all reports it is evident that the risk involved is not as great as the risk of full-term delivery. One is not "balancing risks" between the fetus and the mother, as one considers the chances of fetal defect and counsels the parents in this regard.

While the past 10 years has seen great progress in the ability to establish intrauterine diagnoses with relative precision, much of this counselling still has to be carried out on the basis of statistical correlations of the type discussed above. The exact lesion (or absence of lesion) of the individual fetus is not determined but the general magnitude of the fetal risk as an approximate percentage is known. Some of the most evident of these situations of statistical correlations are the following:

A. The production of a defective child. As soon as any given couple produces a baby with congenital abnormalities, their chances of having another abnormal baby rises from 3 percent to 17 percent. This gross figure has been calculated without regard for the nature and origin of the defects involved, and hence serves more as a basis for worry than as a basis for intelligent future action.

Such approximate predictions, of course, can be rendered more accurate by a precise diagnosis of the defect in the first child. The mode of inheritance for many lesions, whether as autosomal dominants or recessives, is well understood. There is no need to repeat here the chances of repetition for each of these individual entities. McKusick's excellent compendium (1) of congenital lesions should be consulted but the essential point is that *a precise diagnosis must be established.* Nevertheless, the potential parent has to make a decision on the basis of a percentage risk which is only useful in an approximate sense.

B. Radiation. Any fetus inadvertently exposed to irradiation is at some risk. This risk is directly proportionate to the amount of radiation delivered to the maternal midpelvic plane and is most evident in its impact the earlier in pregnancy it is delivered. In general, over 10 rads should indicate therapeutic interruption (Fig. 1), and under 5 can be safely ignored (2). This leaves a gray zone between 5 and 10 rads where the decision would be influenced by other factors such as parity, desire for the particular pregnancy, previous sterility, etc. In addition to the obligation to counsel accurately a woman who has received an excessive amount of radiation diagnostically, the physician should have the obligation to avoid such situations, and all X-ray requisitions should carry the date of a woman's last menstrual period. Diagnostic X rays in the post-ovulatory period should be deferred except in emergency situations.

C. Maternal disease states. Diabetes has already been mentioned as a maternal disease associated with a definite but small risk of fetal abnormality, but rubella remains the most notorious example. The most frequently quoted figures are 40 percent of fetuses damaged from a maternal attack in the first month of pregnancy, 20 percent for a second-month attack, and 10 percent for an attack in the third month. Hardy's study (3) of babies born

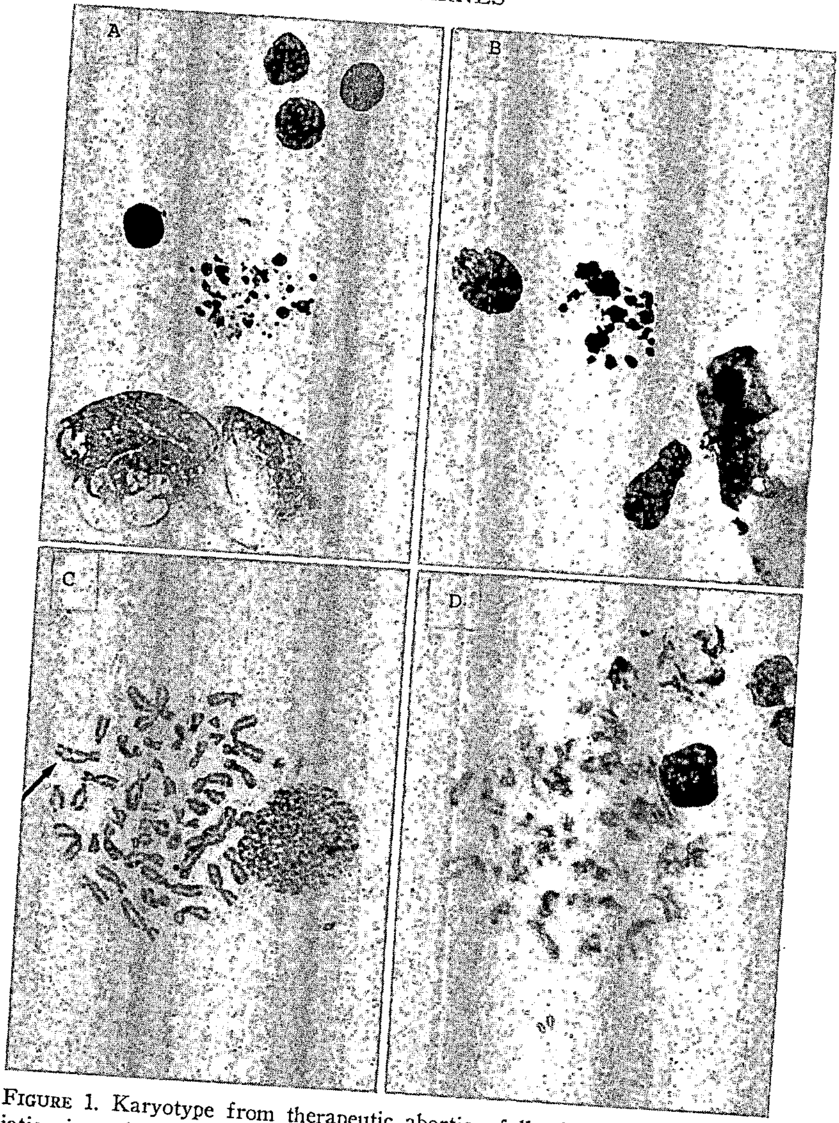

FIGURE 1. Karyotype from therapeutic abortion following excessive diagnostic radiation in early pregnancy. The patient had received a midpelvic dose calculated (including fluoroscopy and scatter) as a minimum of 5 rads and a maximum of 20 (probably between 10 and 15). Since this was in an Army Hospital, she was piously informed that the "Army doesn't do abortions" and no further therapy offered. There is an evident lesion in the 19–20 pair, characteristic of radiation damage.

A & B: Cells from fetus exhibiting marked vacuolization degeneration and complete karyorrhexis—typical radiation effects.

C & D: Two metaphase plates from placenta; C illustrating a chromatid gap in long arm of Group B chromosome and D showing degeneration of all chromosomes.

in the 1964–1965 epidemic, however, indicated that the virus can have damaging effects much later in pregnancy than originally believed and differing viral strains undoubtedly have varying impacts on fetal well-being. Familiar as the problem of rubella in pregnancy is, it takes on added importance from the fact that nationwide epidemics tend to occur in a 6- to 9-year cycle with the next epidemic predicted to occur in the early 1970's (4).

The diagnosis of rubella on the basis of signs and symptoms is far from absolute. The rash can be confused with rashes seen in other infectious diseases or there may be no observable rash at all. The development of the hemagglutination inhibition test which can give the level of maternal antibodies within a matter of hours has significantly altered the management of this disease and should protect against the production of many damaged babies.

It may seem incredible after the publicity given rubella in pregnancy but many women interviewed in the 1965 epidemic (particularly among low income groups) were unaware of the fact that this mild-appearing disease could be a major catastrophe for their baby. The physician's initial responsibility, therefore, is educational. Since an attack prior to but near conception can have a damaging effect, a history of recent suggestive episodes should be obtained. A baseline determination of antibody level should be carried out, and those without antibodies given special warnings. Women with antibody levels above 1:300 should have a repeat within two weeks and a significant change in level, whether up or down, should be taken as evidence of recent infection. The susceptible should have a repeat antibody level at the 10th or 12th week of pregnancy. From these determinations those at major risk can be identified and pregnancy termination can be offered. The "odds and the stakes" principle applies most definitively here, and when in doubt, abortion should be recommended and after stabilization of the antibody levels, a new pregnancy undertaken.

D. Drug and chemical exposure. As rubella is the type example for damaging maternal diseases, thalidomide is the prime example of maternally ingested substances which can seriously damage the fetus. There are, however, many drugs on the American market which can have a deleterious effect on the conceptus, aminopterin being another startling example.

The difficulty with using such chemical exposure as an indication for abortion rests largely on the fact that the epidemiology of the resultant fetal damage is difficult to determine. Isolated episodes of abnormalities associated with individual drugs are recorded, but establishing a frequency curve (or, indeed, causation versus coincidence) is difficult. In addition, the literature on animal experimentation is of limited value. Species specificity is such that the only test animal which will reveal teratogenicity in man is man himself. Continuous monitoring of defective children is essential to identify the new drug threats which will undoubtedly appear.

Again it is not the purpose of this chapter to record in detail all the potential teratogens which are currently known; such lists are available elsewhere (5, 6). In the event of a major identifiable exposure, however, it is probably better to advise interruption. A lifetime of a damaged child is not worth a "wait and see" policy.

In addition to the absence of careful studies giving dose-risk relationships and specific time intervals for the human fetus, much of the difficulty stems from the fact that many of the chemical and drug exposures take place without the patient being fully aware of them. The habitual nature of many home remedies (i.e., the morning dropperful of vasoconstrictive nose drops) may result in their not being consciously identified. Food additives or contaminants (i.e., the implanting of pellets of sex steroids or of busulfan—a gamete poison—to caponize fowl) which are not always known to the consumer, or atmospheric pollutants (carbon monoxide from automobile exhausts) which cannot be measured, all constitute potential hazards which are not usually identified.

LSD represents an exception. The patient is, in most circumstances, aware when she or her partner have been LSD users, and many have heard that it is a "chromosome poison." The situation with respect to the consumption of lysergic-acid-diethylamide in early pregnancy, however, is far from clear.

The question was first raised by Zellweger, McDonald & Abbo (7) who reported a child with the typical picture of the unilateral fibular aplastic syndrome born to a mother who had taken LSD in early pregnancy. Chromatid breaks were found in the peripheral white blood cells of both the mother and father (also an LSD user) as well as the child. These reported chromosome breaks resembled those seen in fetuses receiving irradiation in the first trimester. Later, two additional cases (8, 9) were reported where the mother had used LSD in early pregnancy and the baby displayed skeletal abnormalities. In one of these, no chromosome studies were carried out, whereas in the other a careful examination of 31 metaphases failed to reveal broken chromosomes. Subsequently conflicting reports—not all of them scientifically objective—have appeared concerning the possible teratogenicity of LSD, and at the present time it can only be concluded that it is not positively known whether or not such exposure is capable of producing malformations or with what degree of consistency.

Whether exposure to LSD would be considered a reasonable fetal indication for pregnancy interruption under such circumstances would seem to be almost elective. Since epidemiologic studies are sorely needed in this area, it is to be hoped that the fetal blood and tissues would be carefully karyotyped in all instances where interruption is chosen.

If the hazards discussed above represent the major group of statistical correlates, there is also a host of conditions which are susceptible to more precise diagnosis. This field of endeavor has expanded almost beyond belief in the past decade.

While tapping the amniotic cavity has been carried out for some years to measure intrauterine pressures and record the progress of labor, it was Bevis in 1952 (10) who first applied this technique systematically to gain information as to the state of the fetus. Bevis' original interest was with bilirubin in Rh disease but subsequent workers have extended amniotic fluid diagnoses to cellular components, karyotypes, and enzymatic content.

Severe Rh incompatibility as an indication for termination of pregnancy, however, is usually based on the history and maternal antibody levels rather than amniotic fluid spectrometric analysis. The latter procedures are useful in the management of the last trimester of an afflicted pregnancy but, in general, are not revealing enough to be decisive in the earlier weeks when abortion can be safely considered.

On the other hand, a sensitized Rh-negative woman with a history of intrauterine deaths at progressively earlier times who becomes inadvertently pregnant may well represent a fetal indication for interruption based on this history alone. Unfortunately, most workers have been unable to substantiate Ducos' claim (11) that the fetal Rh type can be determined, but from the husband's apparent homozygosity and the degree of elevation of the maternal antibodies, the presence of an Rh-positive fetus can be assumed.

The long and, at times, entertaining history of man's efforts to predict the gender of the unborn child has been well reviewed by Cederqvist & Fuchs (12). With the identification of the sex chromatin body as being a peculiar characteristic of the nucleus of cells from females, antenatal sex determination moved into the realm of the practical.

Transabdominal amniocentesis can usually produce sufficient fluid from the 14th or 16th week of gestation on. Sterile precautions should be observed and the abdomen carefully prepared, including, where necessary, shaving. The bladder should be empty and a 14-gauge 4-inch needle introduced in the midline directly above the pubic bone and angled about 30 degrees cephalad. Constant gentle suction should be applied as the needle advances and in the event blood is encountered, the needle should be withdrawn and another location tried. There is no apparent harm to placental puncture (or, indeed, as Alvarez has shown, to placental needle biopsy) as long as the fetal plate of the placenta is not violated.

Between 5 and 10 cc of fluid can be aspirated which is centrifuged and the cellular sediment spread out and stained. By the 12th week of pregnancy, a sufficient number of cells should be obtained to make the diagnosis reliable. The chromatin body is readily stained with a variety of stains: it is Feulgen-positive and takes up the green stain of methyl green-pyronine mixtures. It can be demonstrated with such basic dyes as cresyl echt violet, thionine, and hematoxylin.

Makowski, Prem & Kaiser (13) found the mean incidence of sex chromatin in amniotic fluid cell nuclei from a female fetus to be several times that found in the presence of a male fetus. The minimum incidence with a female fetus present was twice the maximum incidence with a male fetus. This gives a high degree of discrimination and if a sufficient number of cells are obtained the diagnostic accuracy should be close to absolute.

While the risk of this procedure is minimal, it is sufficient that amniocentesis should not be used to satisfy idle parental curiosity. Its principal application as far as the topic at hand is concerned, of course, is the antenatal determination of sex-linked defects. These are not common (hemophilia, some progressive muscular dystrophy, and sex-linked Hunter's syndrome) but their severity is such that eugenic measures to eliminate them are justified. In sex-linked recessive disorders it is almost always the male that is afflicted. The only pitfalls in this approach to intrauterine diagnosis would be a female with Turner's syndrome who would have the cell pattern of a normal male, and males with Klinefelter's syndrome who would give a cell pattern which would be diagnosed as normal female. Fortunately, these forms of gonadal dysgenesis are quite rare.

In 1956, Coombs, Bedford & Rouillard (14) reported a technique for detecting blood group antigens from epidermal cells, and subsequently that same year Fuchs and co-workers (15) applied a modified technique to cells from amniotic fluid. Hence, the ABO group of the fetus can be determined. Unfortunately, as mentioned above, these or similar techniques cannot be used for determining subgroups (including Rhesus factor). At the present time the only known ABO blood group-linked hereditary disease is nail-patella syndrome. This might not, of itself, indicate pregnancy interruption but the parents should be informed and given a voice in the matter.

In 1966 several groups of workers reported culturing fetal amniotic cells in sufficient quantity to permit karyotyping. While few laboratories can boast 100 percent success with this procedure, nevertheless, chromosomal abnormalities can be identified in the great majority of cases where they are present. A D/D translocation carrier has been identified at 17 weeks gestation. Nadler (16) and others have identified antenatally the D/G fusion chromosome characteristic of Down's syndrome. Bartholin-Patau's syndrome (13 trisomy) and Edwards' syndrome (18 trisomy) have not been reported to date but should be equally well diagnosable in the fetal state. It is entirely possible that karyotyping the fetus in all pregnancies in women over 40 would be justified.

When cells from patients with mucopolysaccharide storage disorders are cultured and then stained with toluidine-blue 0, intracellular metachromatic granules are demonstrated. This phenomenon is also demonstrable with fetal cells so that Hurler's, Hunter's, and Sanfilippo's syndromes should be diagnosable, as well as cystic fibrosis. Morquio's syndrome sometimes, but not always, displays such metachromasia. Juvenile familial amaurotic idiocy and Gaucher's disease are storage diseases rather than mucopolysaccharidoses, yet the cells from these cases often display the metachromatic phenomenon.

The practical limitations to such studies of cultivated amniotic cells are obvious since there is no way of differentiating a heterozygote from a homozygote. Whether or not determining the mucopolysaccharide content of the amniotic fluid would assist in making this distinction is not known. Furthermore, the test is difficult enough that it is not for the occasional labora-

TABLE 1. Enzymes Demonstrable in Cultured Normal Amniotic Cells

Enzyme	Potential Disease Application
Acid phosphatase	Fatal familial metabolic disorder
Alkaline phosphatase	Hypophosphatasia
Argininosuccinase	Argininosuccinicaciduria
Arylsulfatase A	Metachromatic leukodystrophy
"Branching enzyme"	Type IV glycogen storage disease
α-Glucosidase	Pompe's disease (GSD 11)
α-Keto isocaproate decarboxylase	Maple Syrup Urine disease
β-Glucuronidase	"1-Cell" disease
Cystathionine synthase	Homocystinuria
Galactose-1-phosphate uridyl transferase	Galactosemia
Glucocerebrosidase	Gaucher's disease
Glucose-6-phosphate dehydrogenase	G-6-PD deficiency
Hexosaminidase	Tay-Sachs
Hypoxanthine-guanine phosphoribosyl transferase	
Lactic dehydrogenase	
Phytanic acid oxidase	Refsum's disease
6-Phosphogluconic dehydrogenase	
Sphingomyelinase	Neimann-Pick disease
Valine transaminase	Hypervalinemia

tory. Particularly in fibrocystic disease consistent results are difficult to obtain in the time available.

The most recent extension of the prenatal diagnosis of hereditary disorders is the study of enzymes in cells from amniotic fluid. In general, these have been cultured, but Nadler & Gerbie (17) have performed biochemical analysis directly without prior cultivation. Table 1 indicates those enzymes and their related diseases which can be diagnosed from cultured amniotic cells. The list of genetic metabolic diseases which can be diagnosed from neonatal skin fibroblast cell cultures is, of course, more extensive but the relative enzyme-specific activity may not always be established in normal fetal tissue by the 18th to 22nd week of gestation.

As Edwards (18) has pointed out, in all amniotic fluid studies the basic problem is one of timing. The hazards of misinterpreting what has been obtained decline throughout pregnancy and are least at birth. On the other hand, the hazards of pregnancy interruption rise slightly but progressively as time goes on and the procedure is unacceptable after 26 or 28 weeks. At 10 weeks the fetus is smaller than the yolk of a hen's egg and so fragile that it falls apart when handled; at 20 weeks the injection of hypertonic saline is required with a prolonged waiting period and uncomfortable labor and delivery involved.

Jeffcoate (19) and later Nichols (20) reported the prenatal diagnosis of congenital adrenal hyperplasia (adrenal-genital syndrome) by measuring the 17-ketosteroids and pregnantriol in the amniotic fluid. Not all workers have been able to confirm this finding consistently, plausible as it should be on physiologic grounds. Those cases in which these steroids were demonstrably abnormal were diagnosed too late to consider pregnancy termination.

Before leaving the subject of those fetal lesions which are diagnosable by amniocentesis, it is necessary to consider the (sometimes vexing) question of Who gets tapped? With laboratory facilities limited and with some slight risk inherent in the procedure, the wholesale antenatal diagnosis of an entire generation is obviously impossible.

Abbo & Zellweger (21) have pointed out that prenatal sex determination should be carried out whenever the mother is a true or probable carrier of the mutant gene of an X-linked disease. This would include Duchene and Becker dystrophy, the X-linked variant of mucopolysaccharidosis, hemophilia A and B, Lasch-Nyan hyperuricemia, and Lowe's syndrome. To this group, of course, should be added all normal women whose husbands are carriers of X-linked diseases.

For the determination of karyotype, Abbo & Zellweger advance the concept that any woman who carries a chance of over 1 percent of her fetus being aneuploid should have a diagnostic amniocentesis as early as feasible. Precise risk figures for aneuploidies other than Down's syndrome have not been worked out with accuracy. Nevertheless, the authors suggest the following five categories:

1. 21-trisomic women, or women with a mosaicism which includes a 21-trisomic cell population. Actually, since 21-trisomic women carry a 50 percent risk of having a trisomic zygote, they should have the opportunity to consider surgical sterilization and adoption.
2. Women with balanced D/G, G/G, or other translocation. Since the 21/21 translocation carries a 100 percent chance of a mongoloid child, surgical sterilization and adoption should again be offered.
3. Women whose husbands have the translocations mentioned above and, once again, vasectomy should be offered when the percentage of risk approaches to 20 or 25 percent.
4. Women who have, or women whose husbands have, an increased number of satellite associations, presumably predisposing them to a greater risk of nondisjunction.
5. Pregnant women aged 40 and over.

With the exception of the last group, most women with these lesions usually have been identified because they have produced a defective child. Only a tiny fraction of the population has had a karyotype determination as a routine health procedure, and the most frequent indication for such a determination is usually a defective person, either in the sibship or in the progeny. Thus, a defective child becomes the "ticket of admittance" to karyotyping. Under such circumstances, the irreducible minimum of genetic defectives in the population will not soon become zero.

In the past, however, progress in therapy has led to the prospect of increasing the incidence of the disease or of the carrier state. With the identification of a genetic disease entity and improvement in its management, more and more "unsuitable" people reached the reproductive years. Armed with the weapons of prenatal diagnosis and the sensible use of pregnancy termination, such people may now approach parenthood with greater confidence.

Finally, the self-evident and practical point should be stressed that abortion should be accepted and, indeed, the necessary operative permits signed before the diagnostic studies outlined here are launched. The establishment of a diagnosis should become the basis for therapeutic action rather than the basis for further worry and aimless discussion. Indeed, with those studies involving the determination of fetal gender, the uterus should be evacuated within a few hours of the amniocentesis, rather than condemning the patient to additional days of unnecessary worry.

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ANTIBODY VARIABILITY

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INTRODUCTION

The structure (1, 2), evolution (3), genetics (4, 5), and variability (6, 7) of immunoglobulins have recently been extensively reviewed. This review will concentrate on the basis of the variability in amino acid sequence of immunoglobulin polypeptide chains, since this variability seems able to account completely for the enormous variety of possible antibody specificities (8).

Immunoglobulins are unique among well-characterized proteins in their heterogeneity, as is illustrated by the studies of products of many hundreds of immunoglobulin-secreting tumors from humans and mice; although each product is a well-defined, usually homogeneous protein, no two such proteins have been proven identical [with very few exceptions to be discussed (p. 159)]. These "pathologic" immunoglobulins are apparently not themselves aberrant proteins, for both in the structure of individual molecules and in the statistics of their occurrence they resemble randomly selected representatives of the normal heterogeneous immunoglobulin pool (9, 10). In fact, a certain percentage of the tumor-produced proteins possesses demonstrable antibody activity (11, 12) (See p. 162). For this reason, much of the work to be discussed has been carried out on homogeneous tumor products, but the results are quite consistent with more recent structural studies on normal immunoglobulins (13–17) and antibody preparations (p. 162).

IMMUNOGLOBULIN STRUCTURE

The characteristic four-polypeptide-chain structure of an immunoglobulin molecule is shown in Figure 1. Pairs of identical light and heavy chains (of molecular weights 25,000 and 50–70,000) are linked by disulfide bridges. The distinctive feature of immunoglobulin chains is that the amino terminal half (of a light chain) or quarter (of a heavy chain) has a unique sequence, differing usually in at least ten positions out of just over a hundred from all other chains. This amino terminal portion is called the variable region (V-region)[1] of the chain. In contrast, the remainder of the

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[1] The terminology we have used is as far as possible that recommended by the World Health Organization (36–39).

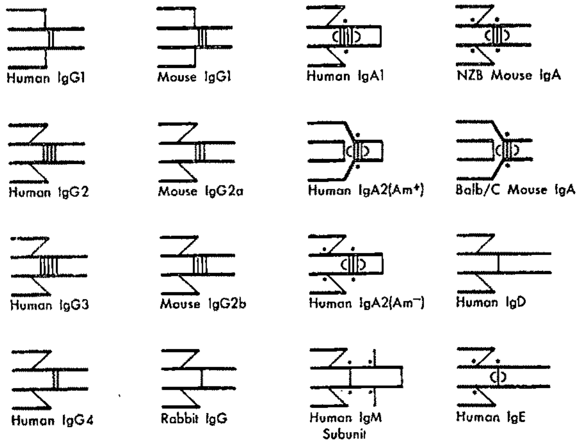

FIGURE 1. Distributions of interchain disulfide bridges (thin lines) joining light and heavy chains (short and long thick lines) in different immunoglobulin classes and subclasses. The positions of the bridge are taken by comparison with homologous sequences in human IgG1 molecules (18). In some cases it is not ruled out that the marked bridges are labile intrachain rather than interchain bridges.

The exact number of inter-heavy chain disulfide bridges (bracketed) in IgA molecules is uncertain, as are the positions in the heavy chain of the bridges marked with an asterisk. The bridge involved in covalent dimerization of IgA molecules has not been established. Am^+ and Am^- are allotypic forms of human IgA 2 molecules.

Data from references 7, 19–21 (Human IgG); 22, 23 (Mouse IgG); 24 (Rabbit IgG); 25–27 (Human IgA); 28–30 (Mouse IgA); 31, 32 (Human IgD); 33–35 (Human IgM).

chain (the constant or C-region) is identical in immunoglobulin molecules of a given class and subclass, apart from the occurrence of genetically polymorphic forms. The two regions appear to be under separate genetic control (see next section).

Differences in the C-regions of heavy and light chains may be genetic polymorphisms ("allotypic" differences) or may define different "classes" or "types" of immunoglobulin present in all normal individuals. Immunoglobulin types K or L[1] are defined by the possession of light chains of type κ or λ, and the immunogloblin classes G, M, A, D, and E are defined by the possession of heavy chains γ, μ, α, δ, and ε. Any heavy chain class may be combined with any light chain type.[2]

It appears likely that the major chain types and classes arose by gene duplication (42, 43) relatively early in evolution, and that all mammalian species will be shown to possess classes of immunoglobulins homologous to the human classes. Thus, most or all mammalian and avian species (44) and possibly alligators (45), paddlefish (46), and some shark species (47–49) have chains homologous to human κ and λ chains (on the basis of limited sequence data). Similarly, bullfrogs (50) and higher species (3) have chains probably homologous to μ and γ chains, although in several lower species (e.g., sharks) only one heavy chain class has been identified (51–54). IgA-like molecules have recently been described in secretions of a large number of mammalian species (3, 55–57) although the amounts present in serum may be low. Their existence in other animal classes is still controversial. Molecules with properties similar to those of human IgE (reaginic antibody) (58, 59) have been found in rabbits (60), dogs (61), and monkeys (61, 62) and probably also rats (63) and mice (64); with sufficiently sensitive assays, IgE and IgD-like immunoglobulins may be discovered in a large number of species. The apparent lack of biological properties (complement fixation, skin sensitization) of IgD (65–67) may make its detection difficult.

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[2] δ chains appear to combine preferentially with λ light chains (40, 41).

More recent evolution by gene duplication of the class-specific (C-region) genes has given rise to subclasses of heavy chains (and possibly subtypes of light chains); thus, humans have four genes for γ-class chains (γ1, 2, 3 ,and 4 chains) (68) and at least two each for α1 and 2 (69–72) and μ1 and 2 (73) subclasses. As they are products of recent evolution, the subclasses of other species are not necessarily related to the human subclasses, although the nomenclatures (and in some cases the biological properties) are similar. For example, in some species, electrophoretically fast-moving IgGs (IgG4 in humans; IgG1 in mice and guinea pigs) mediate homologous passive cutaneous anaphylaxis reactions (74–76) but do not fix complement well (77–79); however, in other species the opposite is the case (80). The subclasses of all these species most probably have independent evolutionary origins: this is suggested both by structural (81, 82) and immunogenetic (83, 84) studies.

Light chain C-region "subtypes" are probably uncommon, as only human λ chains have thus far been classifiable (by serological and chemical methods) into "subtypes"; these are the Oz^+ and Oz^- λ chains (85, 86), both present in normal humans (87), which appear to differ only by a single amino acid substitution in the λ chain C-region (86). The two types of chain are probably also a result of recent gene duplication.[3] It is possible that a similar situation exists in the case of λ chains in some rabbits (88).

Immunoglobulin light and heavy chains are usually joined by interchain disulfide bridges (Fig. 1). Two lines of evidence suggest that the structure adjacent to these bridges may be of importance for the biological properties of the molecule. First, different classes and subclasses, whose biological properties may be different, have widely different interchain bridge patterns (21–23). Secondly, at least in some cases, reduction causes a loss of certain biological activities, for example, complement fixation (89, 90). However, studies with isolated biologically active fragments suggest that the bridges are not directly involved in the complement fixation site (91, 92); further, the number and position of bridges are not directly correlated with biologic activity (23). It is possible that the bridges assist in activating this site (in the Fc fragment) by a conformational change upon antigen binding or aggregation of the antibody molecules. The evidence for the biological role of conformational changes in antibodies on antigen binding is not yet conclusive and has been reviewed by Metzger (93). The complement (C1) binding site is thought to be located in the amino terminal portion of the Fc fragment (91, 92). A small number of structural differences between complement-fixing and nonfixing molecules occurs in this region (82).

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[3] Recent gene duplication is a feature of other mammalian systems, including the hemoglobin genes (see Schroeder et al 1968. *Proc. Nat. Acad. Sci. USA* 60:537).

The structure of IgM and IgA molecules is based on a four-chain unit similar to that of IgG (Fig. 1). An IgM molecule includes five four-chain units linked by disulfide bridges (33). The units are not linked by the cysteine penultimate to the μ chain C-terminus (34, 35), as had been suggested on grounds of homology between μ and light chains by Singer & Doolittle (43), and they may be linked through an extra "joining" peptide chain (J-chain, see below).

The four-chain units of IgA molecules may exist as such or polymerized in serum (94, 95), or as dimers combined with a "secretory component" in external secretions such as colostrum, saliva, and gastric juice (96–98). The secretory component has so far been difficult to characterize (98, 99). The secretory immune system is responsible for most of the body IgA production; it appears to be relatively independent of the IgG- and IgM-producing lymphoid tissues (56). However, human IgA myeloma rarely if ever involves, for example, the gastrointestinal tract (we have looked for such problems in the 54 IgA myelomas which we have seen in the past ten years, but we have never observed them). IgA myeloma proteins and alpha heavy chain disease proteins (see p. 156) are not associated with the secretory component, which is probably not synthesized by immunoglobulin-producing cells (100).

In some species (e.g., cattle, sheep, pigs, and horses) IgG-like molecules are the predominant immune globulins in secretions (3, 56, 101). Nevertheless, the IgA found in these species was selectively concentrated in their secretions (55). Such IgG molecules are not associated with a secretory component, although it seems probable that the extra component is necessary for normal secretion of IgA molecules.

An extra chain type (J-chain) has been described in polymeric IgA molecules (both in serum and secretions) and in some, perhaps all, IgM molecules (102). J-chain has a molecular weight of around 20,000 (102), and is quite distinct from the secretory piece (molecular weight about 70,000)

(103). These results provide the possible explanations for a number of puzzling findings, which included: the presence of allotypic markers from three unlinked loci on rabbit serum IgA (104) and possibly IgM molecules (105); the findings of diverse molecular weights for the "secretory component" (97, 103) and for IgM subunits (106); and the proposal that three light and two heavy chains composed one such subunit (107, 108).

If this last result is correct, J-chain may be present in a ratio of one molecule per four-chain IgM immunoglobulin subunit, as an extra disulfide-bonded chain (102). It is of interest that the asymmetry implied by this arrangement may be reflected in asymmetry of the antigen-binding sites of IgM molecules (109–112).

SEPARATE GENETIC SYSTEMS FOR C- AND V-REGIONS

The presence of Mendelian genetic markers, identified chemically or serologically, on several C-regions indicates the presence of a single gene for the corresponding C-region of an immunoglobulin chain of given subclass. Such well-established genetic markers exist, for example, on human κ, $\gamma 1$, $\gamma 2$, and $\gamma 3$ (5, 68), and $\alpha 2$ (113, 114) chains, and have recently been described on $\gamma 4$ chains (115). The human κ chain markers *Inv* [1, a] or [1, 2] and *Inv* [b] or [3] are associated with a single amino acid interchange (126, 127), while the *Gm* [a+] [1] and *Gm* [non-a] [−1] alleles in $\gamma 1$ chains are associated with a double interchange (18, 21, 116, 117). Other interchanges possibly associated with allelic heavy chain C-region markers are shown in Table 1.

Light chains.—Genetic markers on the V-regions of human immunoglobulin chains have not been described. Nevertheless, a study of the amino acid sequences of many variable regions indicates that separate genetic systems are involved in V-region and C-region synthesis (128–132); thus, although there is one κ-chain C-region gene, in contrast the group of κ-chain V-region sequences can be divided into at least three main "subgroups" (See Table 2) (130) each of which presumably originates from a separate gene or genes. Apparently there exists a mechanism for integrating C- and V-region genetic information, probably operating on DNA sequences (See p. 156).

Similar examination of λ chain V-regions shows the presence of at least four or five subgroups of λ chain V-region sequences (6, 132, 150, 151). The prototype subgroups are shown in Table 2; examples of variation of individual proteins of the same subgroup are also shown in Table 2 for both κ and λ chains. Representatives of each subgroup are probably present in each normal serum (131, 152, 153), while only one of the subgroups is represented in any given myeloma protein. Members of any κ subgroup can combine with either *Inv* 1, 2, or *Inv* 3 κ chains (154), and members of any λ subgroup can combine with Oz^+ or $Oz^-\lambda$ chains (132, 151, 155). Similarly,

TABLE 1. Amino acid interchanges probably associated with allotypic differences in immunoglobulin heavy chains. Residue numbering is by homology with human $\gamma 1$ chains (18).

Chain	Residue number	Amino acid residue or function	Associated allotype	Reference
Human $\gamma 1$	356, 358	Asp, Leu Glu, Met	*Gm*[a] [1] *Gm* [non-a] [1-]	18, 21, 116, 117
Human $\gamma 1$	214	Arg Lys	*Gm* [f] [4] *Gm* [f-] [4-]	118, 119
Human $\gamma 3$	436	Phe Tyr	*Gm* [b] [5] *Gm* [g] [21]	120
Human $\gamma 3$	296	Phe Tyr	*Gm* [b] [5] *Gm* [g] [21]	121
Human $\alpha 2$	131	Bridge to light chain No bridge	*Am* [2-] *Am* [2+]	26
Mouse α	131	Bridge to light chain No bridge	In NZB mice In BALB/C mice	29, 30
Mouse $\gamma 2a$	135	Ser Thr	In C3H mice In BALB/C mice	22
Rabbit γ	225	Met Thr	*Ad* 11 *Ad* 12	122, 123
Rabbit γ	1–34	Multiple differences	*Aa* 1, *Aa* 3	123–125

in rabbits, κ chains with characteristically different V-regions are found in association with all C-region allotypes (156, 157).

These genetic and amino acid sequence studies strongly suggest that each light chain is the product of two separate genes, one for the C-region and the other a gene which is only one of a "group" of κ chain or λ chain V-region genes. A minimum number of genes (one for each subgroup) for light chain V-regions can be derived from these studies and Figure 2 illustrates this "minimum genetic system" for light chains. There is suggestive chemical evidence of similar V-region subgroups in rabbit (158), guinea pig (159), and mouse κ light chains (129, 160–162). The significance of differences between "corresponding" subgroups in different species (163) is discussed below (p. 158).

TABLE 2. Subgroups of variable region of κ, λ and heavy chains. Nomenclature is that recommended by the World Health Organization (39). Light chain sequence data are more fully presented in References 1–7, 133 and 134, and are here selected to show the presence of further divisions of each basic sequence (see text). Heavy chain data are from References 135–149. Solid lines indicate identity to basic sequence of corresponding subgroup. Glp indicates pyrrolidone carboxylic acid; Glx indicates glutamic acid or glutamine. Gaps have been arbitrarily inserted in λ and heavy chains to show their evolutionary relationship to each other and to κ chains.

KAPPA CHAINS					5					10					15					20
VκI Basic Sequence:	Asp-	Ile-	Gln-	Met-	Thr-	Gln-	Ser-	Pro-	Ser-	Ser-	Leu-	Ser-	Ala-	Ser-	Val-	Gly-	Asp-	Arg-	Val-	Thr-
Protein Ag	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Roy	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Eu	—	—	—	—	—	—	—	—	—	Thr	—	—	—	—	—	—	—	—	—	—
Mon	—	—	—	—	—	—	—	—	—	Thr	—	—	—	—	—	—	—	—	—	—
Bel	—	—	—	Leu	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Lux	—	—	—	Leu	—	—	—	—	—	Phe	—	—	—	—	—	—	—	—	—	—
VκII Basic Sequence:	Glu-	Ile-	Val-	Leu-	Thr-	Gln-	Ser-	Pro-	Gly-	Thr-	Leu-	Ser-	Leu-	Ser-	Pro-	Gly-	Glu-	Arg-	Ala-	Thr-
Protein Nig	Lys	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Rad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asp	—	—	—
Cas	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Asp	—	—	—
Smi	—	—	—	—	—	—	—	—	Ala	—	—	—	—	—	—	—	—	—	—	—
Win	Asp	—	—	—	—	—	—	—	Ala	—	—	—	—	—	—	—	—	—	—	—
VκIII Basic Sequence:	Asp-	Ile-	Val-	Met-	Thr-	Gln-	Ser-	Pro-	Leu-	Ser-	Leu-	Pro-	Val-	Thr-	Pro-	Gly-	Glu-	Pro-	Ala-	Ser-
Protein Tew	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mil	—	—	—	Leu	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LAMBDA CHAINS																				
VλI Basic Sequence:	Glp-	Ser-	Val-	Leu-	Thr-	Gln-	Pro-	Pro-	GAP-	Ser-	Val-	Ser-	Gly-	Ala-	Pro-	Gly-	Gln-	Arg-	Val-	Thr-
Protein HS92	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
HS78	—	—	Ala	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Thr	—	—
HBJ7	—	—	—	—	—	—	—	—	—	—	Ala	—	—	Thr	—	—	—	Gly	—	—
HBJ11	—	—	—	—	—	—	—	—	—	—	Ala	—	—	Thr	—	—	—	—		

TABLE 2—(*Continued*)

KAPPA CHAINS		5 10 15 20
VλII Basic Sequence:		Glp-Ser-Ala-Leu-Thr-Gln-Pro-Ala-GAP-Ser-Val-Ser-Gly-Ser-Pro-Gly-Gln-Ser-Ile-Thr-
Protein HS68		————————————————————————
HBJ8		—————Ala——————————————————
HS86		———Pro——Ala———————————————Glu————
VλIII Basic Sequence:		Tyr-Val-Leu-Thr-Gln-Pro-Pro-GAP-Ser-Val-Ser-Val-Ser-Pro-Gly-Gln-Thr-Ala-Ser-
Protein Kern		——Ala—————————————————————Val
X		——Asp——————————————————————
VλIV Basic Sequence:		Glp-Ser-Ala-Leu-Thr-Gln-Pro-Pro-GAP-Ser-Ala-Ser-Gly-Ser-Pro-Gly-Gln-Ser-Val-Thr-
Protein Bo		————————————————————————
HBJ2		————————————————————————
VλV Basic Sequence:		Ser-Glu-Leu-Thr-Gln-Asp-Pro-GAP-Ala-Val-Ser-Val-Ala-Leu-Gly-Gln-Thr-Val-Arg-
Protein Sh		————————————————————————
HEAVY CHAINS		
VHI Basic Sequence:		Glp-Val-Gln-Leu-Val-Glu-Ser-Gly-GAP-Ala-Glu-Val-Lys-Lys-Pro-Gly-Ala-Ser-Val-Lys-
Protein Eu γ1	(135)	—————————Gln————————————————Ser—————
Ste γ1	(136)	———His——————Ser——————————————————Met——
Ca γ1	(137)	—————————Gln——————————Arg——————————
Zuc γ3 HCD[a]	(138)	—————Val——————————Asp-Leu-Val—————Gly.——
VHII Basic Sequence:		Glp-Val-Thr-Leu-Arg-Glu-Ser-Gly-GAP-Pro-Ala-Leu-Val-Lys-Pro-Thr-Gln-Thr-Leu-Thr-
Protein Daw γ1	(139)	———————————————————Arg——————————
Cor γ1	(139)	————————————————————————————
Ou μ	(140)	——————Thr————————————————Lys——Pro————
He γ1	(141)	——————Lys——Asn——————Thr———————————

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[a] Heavy Chain disease proteins.

						5					10					15					20
VHIII Basic Sequence		Glu	Val	Gln	Leu	Val	Glu	Ser	Gly	GAP	Gly	Gly	Leu	Val	Gln	Pro	Gly	Gly	Ser[b]	Leu	Arg
Vin γ4	(142)	—	—	—	—	—	—	—	—	—	—	—	—	Ile	—	—	—	—	—	—	—
Hi γ HCD[a]	(143)	Gly	—	Leu	—	—	—	—	—	—	—	Val	Ser	Ile	?	—	—	—	—	—	
Wat γ2	(144)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
For α1	(144)	—	Ile	—	—	—	—	—	—	—	—	—	—	—	Lys	Gly	—	—	—	—	—
Low α2	(145)	—	—	—	—	—	Glx	—	—	—	—	—	—	—	?	—	—	—	—	—	—
Jor α1	(145)	Glx	—	Glx	—	—	Glx	—	—	—	—	—	—	—	Glx	—	—	—	—	—	—
Ha α	(146)	—	—	—	—	—	—	—	—	—	—	—	—	—	Gly	—	—				
Wo μ	(146)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Na μ	(146)	—	—	—	—	—	—	—	—	—	—	Ala	—								
Till μ and γ	(147)	—	—	—	—	Leu	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sha ε	(148)	—	—	—	—	Met	—	—	—	—	—	—	Val	—	Lys	—	—	—	—	—	—
Wei γ	(145)	Asx	—	Glx	—	Met	Glx	—	—	—	—	Ala	—	—	Lys	—	—	Glx	—	—	—
VHIV Basic Sequence:		Glp	Ser	Val	Leu	Asx	—														
Dos μ	(149)	—	—	—	Ala	—															
Dau μ	(149)	—	—	—	—	—															
Bus μ	(149)	—	—	—	—	—															
Bal μ	(149)	—	—	—	Ala																
Re μ	(146)	—	—	Ala	—																

[b] This residue may be threonine in proteins Low, Jor, and Wei.

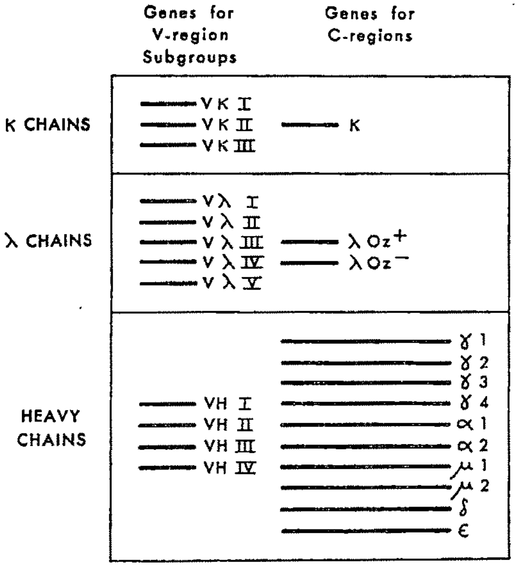

FIGURE 2. Minimum genetic systems for human immunoglobulin chain genes. Each box encloses a system of genes which are probably genetically linked (see text). Each bar represents at least one gene; the numbers of genes in each box are minimum numbers, except in the cases for which genetic markers (presumably carried by a single gene) have been described (κ, γ1, γ2, γ3, γ4, and α2 chains). The numbers of V-region genes are further discussed in the text. One V-region gene and one C-region gene contribute to each immunoglobulin chain. Each gene is labeled by the name of the corresponding protein product or products.

The presence of further divisions of subgroups (shown in Table 2) in both κ and λ chain V-region sequences (6, 7 ,131) has been emphasized (6). These divisions are interpreted as resulting from classification of products of very large numbers of V-region genes (of the order of 10^4 or 10^5), and a minimal classification which takes these results into account must include at least four or five genes for κ chain V-regions (Fig. 2). The existence of such "sub-subgroups" is also suggested by the occasional length differences found between proteins apparently of the same subgroup (2, 151).

Heavy chains.—The genetic control of heavy chain synthesis differs in important ways from that of light chains. Light chain V-regions are grouped into separate κ and λ "groups" (and then further into subgroups). κ Chain V-regions are not found associated with λ C-regions (and vice versa). In contrast, all heavy chain V-regions seem to belong to a single heavy chain group, whose members may be associated with any heavy chain C-region (γ, μ, α, etc.).

So far, three subgroups[4] have been well defined (139–146), and there is evidence for a fourth (149) within this heavy chain V-region group (Table 2). The variability of this group of sequences seems to be similar in character to that seen in light chains. Additional data have not borne out the suggestion (164) that heavy chains are more variable than light chains. The "minimum genetic system" for human heavy chains is shown in Figure 2. Similar subgroups may exist in mice (165), but the major amino terminal sequences for rabbit γ chains (124) cannot easily be fitted into the main human subgroups.

The conclusion that heavy chain V-region subgroups may be "shared" by different C-regions was first suggested by the findings of similar or identical genetic (allotypic) markers associated with variable regions of rabbit γ, μ, α and ε (166–168) chains and of cross-reaction between some human IgG and IgM proteins (169–171); it has recently been confirmed by direct comparison of sequences of rabbit γ and α (172) and human γ (139, 141), μ (140), α (144–146), and ε (148) chains.

Other findings emphasize the special nature of the heavy chain "group" of variable region genes. The first is the finding that the κ, λ, and heavy chain constant region genes probably form three separate (unlinked) genetic groups. [The evidence for this is not complete, but in mouse and man, all genetically polymorphic heavy chain genes are linked (68, 113, 173), while in the rabbit, the γ chain and κ chain (174, 175), and probably λ chain C-region genes are all unlinked (88, 176)]. This C-region gene grouping parallels the grouping of V-regions discussed above. The further findings (177–181) that constant and variable regions of rabbit γ chains are genetically linked has led to the speculation that only linked C-region genes may share members of a V-region gene group. In other words, the gene groupings of Figure 2 may correspond roughly to actual chromosomal groupings.

Further support for this hypothesis comes from experiments designed to test whether, in a heterozygous rabbit, maternal heavy chain C-region genes may associate (to form protein products) with paternal heavy chain V-region genes, and vice versa (181). Little or no such somatic interchromosomal exchange appears to take place (182, 183); thus, the integration of C- and V-region genetic information apparently operates on information contained in a single chromosome, and does not involve an "episome" capable of transferring information from one chromosome to another.

Of special interest is the data suggesting that during the immune response molecules with different C-regions but identical V-regions are synthesized. Nossal and his co-workers (184) suggested that individual normal cells could switch from IgM to IgG antibody synthesis while maintaining antibody specificity. Later Oudin & Michel (185) observed that antibodies of a given idiotype[5] were sometimes homogeneous early in the response (IgM) but at least biclonal (IgM and IgG) at later stages. Strong evidence for a mechanism of this sort comes from work on the rare human patients with "double paraproteins." Wang et al (145, 186) reported that the light chains of a patient (Til) synthesizing IgM-K and IgG2-K monoclonal proteins appeared to be identical by several criteria including partial amino acid sequence (Table 3). Most interestingly, their data indicated that the variable regions of the Til IgG and IgM heavy chains were also identical for at least the N-terminal 34 residues (Table 3); further, an idiotypic antiserum (not directed against the light chains) reacted in a quantitatively similar fashion with the IgM and IgG proteins (147). Qualitative idiotypic identity (as gauged by agar diffusion studies) of IgM and IgG paraproteins from another patient has also been reported in abstract form by Penn et al (187).

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[4] Controversy exists (even among the authors) as to the most satisfactory nomenclature for heavy chain variable regions. Such nomenclature should take into account the differences in genetic control of heavy and light chain synthesis (see text). For this reason, the term "class" might be considered preferable to "subgroup." Final choice of nomenclature is made by the World Health Organization.

In at least the first of these cases, different cells synthesized the IgG and IgM C-regions (186, 188), although these cells possessed similar idiotypic (V-region) specificities, demonstrable by fluorescence studies (188). It seems possible that a clone of cells originally synthesized only the IgM, and that a switching mechanism took place, in one or more of these cells, which suppressed the C-region gene for the μ chain and simultaneously activated the C-region gene for the γ chain. Alternatively, a clone of nonantibody-producing cells may have differentiated to give separate lines of IgM and IgG-producers. Such genetic mechanisms would be consistent with the well-documented findings that in general one cell makes immunoglobulins of one allotype (189–191) and one C-region class (subclass) and type only (192–194), and of one antibody specificity only (195–198). The first mechanism would explain the observations (184) that some individual cells may apparently switch from IgM to IgG synthesis [or produce both IgG and IgM molecules (193, 194)] during the course of an immune response, but not the results of others who failed to demonstrate such a switch in cell transfer (199, 200) or plaquing (201, 202) experiments. Immunosuppression of IgM synthesis (203), cloning of cultured cell lines producing more than one class of immunoglobulin (204, 205), and the study of antigen receptor molecules (93), may help to distinguish between the alternatives.

One model for such a switchover mechanism is that an episome-like particle (restricted to a particular chromosome—see above) can carry V-region genes and can be integrated [in the manner of DNA bacteriophages (206)] into DNA adjacent to the μ or the γ genes. The notion of DNA

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[5] An idiotypic specificity (174) is an antigenic determinant or set of determinants found only on a single antibody population (e.g., on a rabbit anti-*Proteus* antibody). Such determinants are not found on other immunoglobulins from the same animal, or on similar antibodies from other animals.

TABLE 3. Amino terminal sequences of heavy and light chains of two paraproteins from a single patient (Til) (145, 147). The sequences are compared with corresponding basic sequences for VκII light chains and VHIII heavy chains. Solid line indicates identity to basic sequence. Glx may be glutamic acid or glutamine.

	5 10 15 20
VκII	Glu-Ile-Val-Leu-Thr-Gln-Ser-Pro-Gly-Thr-Leu-Ser-Leu-Ser-Pro-Gly-Glu-Arg-Ala-Thr-
Til (IgG) κ:	——————————————Ala——————————————————Val——
Til (IgM) κ:	——————————————Ala———————————————Glx———Val——
	25 30 35
VκII	Leu-Ser-Cys-Arg-Ala-Ser-Gln-Ser-Val-Ser-Asn-Ser-Tyr-Leu-Ala-Trp-Tyr-Gln
Til (IgG) κ:	———————————————————GAP——————————————Glx
Til (IgM) κ:	——————————————Glx————————GAP——————————————Glx
	5 10 15 20
VHIII	Glu-Val-Gln-Leu-Val-Glu-Ser-Gly-Gly-Gly-Leu-Val-Gln-Pro-Gly-Gly-Ser-Leu-Arg-Leu-
Til γ :	————————Leu——————————————————————————————
Til μ :	————————Leu——————————————————————————————
	25 30
VHIII	Ser-Cys-Ala-Ala-Ser-Gly-Phe-Thr-Phe-Ser-Thr-Asn-Tyr-Met
Til γ :	——————————————————————?-Tyr-Val——
Til μ :	——————————————————————?-Tyr-Val——

integration has received support from studies of heavy chain disease proteins; these are heavy chains [of the γ (207, 208), α (209), or μ (210) class] some or all of which are results of genetic deletions, in at least one case involving parts of both the constant and variable regions (138, 211). This finding is not easy to reconcile with the idea of integration of messenger RNA molecules from separate genes, but is consistent with the DNA-integration model above (143). Such DNA-level integration has been thought to require "recognition sequences" which would be translated into protein at the switch from constant to variable regions (212). This section (of 15 amino acids) is identical in γ1 and γ4 chains, but a region close to it in μ chains has a different sequence (7, 82).

The immune response in general and, if proven, this simple example of specific gene activation (μ to γ) in particular, seem suitable models for the study of mammalian cell differentiation. However, at least two phenomena in the immune response appear to be atypical of normal differentiation: first, the immunoglobulin genes are the only autosomal mammalian genes [with the possible exception of those coding for rabbit low-density lipoproteins (213)] known to be subject to "allelic exclusion" (that is, only one allele of a given locus is active per cell). Second, gross cellular differentiation usually takes place after one or more cycles of cell division (214), but immunocytes undergo a series of well-characterized major changes, preceding cell division, on stimulation by antigen or certain other agents [phytohemagglutinin, concanavalin A, etc. (215, 216)].

BASIS OF ANTIBODY VARIABILITY

To summarize the preceding section, each immunoglobulin chain is the product of two structural genes, one for the V-region and one for the C-region of the chain. Genetic data indicate the presence of a single structural gene for the C-regions of individual chain classes (types) or subclasses (e.g., for human κ and γ1 chains), while sequence data indicate the presence of three groups of V-region genes, for κ, γ, and heavy chains; each "group" must comprise a minimum of four or five structural genes (at least one for each subgroup) (Fig. 2).

Theories concerning the origin of variability have been based on two opposing ideas: that each V-region subgroup comprised one or a few genes which underwent somatic mutation (217) or specialized recombination (218, 219) during development of an individual or, alternatively, that each individual inherited many (more than 10^3) genes for each subgroup (6, 220). It is difficult to obtain direct evidence concerning the number of genes involved. This may eventually be done by DNA/RNA hybridization, but the problems of messenger RNA isolation (221, 222) and hybridization are great.

Arguments for the existence of small numbers of genes for V-regions are (*a*) that rabbits are polymorphic for heavy chain V-region sequences

(123–125), and that the polymorphic forms are apparently inherited in a simple Mendelian fashion (223). (It should be added, however, that since the rabbit immunoglobulins studied are heterogeneous, it is not possible to draw conclusions about gene number with such certainty as has been done, for example, in the case of human hemoglobin chains.) (*b*) During mammalian evolution, there have been periods when V-region subgroups comprised only one or a few genes which underwent mutation. Thus, most rabbit κ chain V-regions have alanine as an amino terminal residue (158, 163); some of these V-regions form a group homologous to some human κ chains, which have an aspartic acid amino terminus. It is difficult to imagine this mutation occurring independently in many genes during the divergence of rabbits and humans. Such species-specific residues also occur in pig light chains (17). (*c*) Cesari et al (224, 225) have described five apparently identical λ chains from mouse myeloma proteins of different origins (11 were examined); these presumably have V-regions which are products of a single germ-line gene. (*d*) It is not necessary to postulate an ad hoc hypermutation mechanism to generate the observed diversity from a few genes: Cohn (162, 226) and Jerne (227) have shown that the diversity can be produced by very stringent "somatic selection" alone (of individual cells which are spontaneously arising mutants). These authors suggested selection for goodness of fit between antibody heavy and light chains (162, 226), or for molecules which mutate to lose a postulated antibody activity against self-constituents (histocompatibility antigens) (227).

Jerne's theory supposes that germ-line antibody genes code for antibodies directed against histocompatibility antigens, and that genes coding for antibodies against each individual's antigens mutate to give altered products. Antibodies which have not so mutated are selected against, and those which have mutated include many variants active against other antigens.

The theory provides a rationale for the genetic linkage between some antibody response genes and histocompatibility antigen genes (228), and for the finding of large numbers of cells responding to histocompatibility antigens in graft-versus-host reactions (229). Some testable predictions have been made: that antihistocompatibility antigen antibodies may be of restricted heterogeneity; that myeloma proteins with this sort of activity should be found; that immune responses may be altered by paralysis of embryos with histocompatibility antigens; and that antibodies made in animals doubly heterozygous (for immunoglobulin and histocompatibility antigen markers) should not be of restricted allotype in cases (228) where the immune response is under genetic control (230). The prediction that human myeloma proteins should have anti-HL/A activity is being tested: of 20 proteins examined, none so far has exhibited such activity (231).

Hood & Talmage (6) have summarized arguments in favor of the existence of many germ-line genes (one for each V-region sequence). (*a*) As more V-region sequences are studied, more "sub-subgroups" are becoming apparent, each of which requires the presence of another germ-line gene. There is no evidence of recombination between genes for different subgroups. (*b*) The observed variation is very similar to the variation resulting from normal evolutionary processes, and it is possible to construct a consistent evolutionary tree including all the V-region sequences (232). (*c*) The existence of "species-specific residues" can be explained by postulating frequent extensive gene multiplication in different species (6, 7). (*d*) Indirect evidence for large numbers of human κ chain V-region genes comes from the work of Capra & Kunkel (233) who established 40-residue sequences of two κ chains from homogeneous antibody preparations (see next section). The preparations were from two separate patients suffering from hypergammaglobulinemic purpura. The sequences were identical to each other but not to any known V-region subgroup basic sequence, although they were clearly derived from subgroup I (by point mutations in at least six positions out of the 40 examined). It seems more likely that the sequences are identical because of derivation from a common germ-line gene rather than because of parallel somatic mutation.

In the light of this evidence for both large and small numbers of V-region genes in different cases, it seems possible that both situations may exist at different stages during the evolution of a subgroup of V-region genes. At frequent intervals, individual V-region genes may undergo extensive gene multiplication and subsequent mutations. After a certain number of these genes had been eliminated by unequal crossing-over, the multiplication phenomenon would be repeated on another single gene. Such an evolutionary scheme has been proposed to account for the fact that certain DNA sequences are found in many (up to 10^6) repeating copies in certain species (234–236), but the basic repeating sequence may be different even in closely related (rodent) species (237, 238). However, there is no evidence yet that immunoglobulin genes are represented among these repeated DNA sequences (239, 240).

According to this idea, human κ and mouse κ chains may have large germ-line gene reservoirs, while there may be very few different mouse λ chain genes. These λ chain genes may be about to undergo extensive gene duplication, or may have been duplicated but not diversified. Thus, it is not necessarily surprising that λ chains make up a very small fraction of mouse light chains; the ideas put forward here are consistent with findings that the relative amounts of κ and λ chains in different species may be very different (44), since the reservoir of variability in each chain type is different in each species.

The evolution of such a multi-V-region gene system seems quite possible. The multigene systems mentioned above consist of repeating short stretches of DNA which, however, are not all identical. In the case of mouse satellite DNA, the repeating stretch is very short (6 bases ?) (241) but may accumulate mutations at a rate calculated to be of the same order as that found for protein sequences (e.g., cytochromes, hemoglobins, immunoglobulin C-regions). The rate of accumulation of sequence differences between human κ light chain subgroups (which contain species-specific residues and therefore have evolved since the human-rabbit divergence) is also within this average range. If immunoglobulin V-region genes constituted 1 percent of the mammalian genome, mutations could accumulate at the rate of approximately one every 50 years since, for the whole genome, the over-all average rate of accumulation of mutations is thought to be perhaps as large as two per year (242).

The individual selection of very many germ-line genes during evolution has seemed problematic. However, such selection appears to be possible if (*a*) individual antibody molecules are of wide specificity (243) and are therefore generally not "silent" for long evolutionary periods; (*b*) blocks of closely related genes, rather than individual genes, are selected for or against; and (*c*) a limited number of silent or functionless genes can be carried for short periods in the genome. The total DNA (6) content of the genome is large enough to support this extra DNA. Such functionless genes would be unexpressed because of further selection by antigen during individual development.

It is of interest that the pattern of mutations suggests that the gene expansion does not take place as a "saltatory" event producing many genes from one in a single individual. Instead, the finding that V-region subgroups can be continually subdivided (Table 2) suggests a gene multiplication taking place over many generations, perhaps continually (counteracted by gene elimination).

This hypothesis of rapid gene multiplication and elimination does not require a special somatic mutation mechanism, and it does not run into the genetic difficulties met by previous germ-line theories, with one exception. This is the difficulty of apparent Mendelian behavior of rabbit heavy chain V-region allotypes; these are carried by 80 to 90 percent of all rabbit heavy chains (242), so that, if only a few genes are present in the germ-line, somatic mutation must operate on them. However, if the allelic sets of V-region genes had only recently become allelic, by *deletion* of different gene sets from originally identical genomes (rather than point mutation), crossing-over might be difficult to detect if many closely linked genes were involved. So far, a biochemical examination of allotypic specificities in large numbers of rabbits has not been carried out. Even if no crossing-over were found, multiple V-regions could still be postulated by the assumption of specific limitations on crossing-over in certain chromosomal regions (7, 218). The finding of allotypic V-region markers in other species (preferably inbred mice in which myeloma proteins can be induced) may help to solve this problem.

RELATIONSHIP BETWEEN VARIABILITY AND ANTIBODY ACTIVITY

Two approaches have been moderately successful in suggesting the portions of immunoglobulin chains involved in antibody activity. The first has been to note the "most variable" stretches of amino acid sequence of pathologically produced molecules without known antibody activity. Two stretches of light chains (residues 25–35 and 89–96) vary extensively in sequence, sometimes even within a subgroup (7, 244, 245), and even differences in length between molecules of the same subgroup have been noted (7). These stretches are connected by a disulfide bridge. A third region, midway between these two (around residue 50) has also been classed as "hypervariable" (244, 245). Although heavy chains appear more important than light chains for combination with antigen (10), the available sequence data on heavy chains are still insufficient for this sort of comparison; but work on normal rabbit γ chains has suggested that a region from residues 85–100 may be more heterogeneous than the rest of the chain (16, 246). Alternatively, affinity labeling of myeloma proteins with antibody activity (247) or of partially purified antibody preparations (248), has been used in several cases and has led to labeling of positions within the hypervariable sections just mentioned. [Position 34 in a mouse anti-DNP myeloma protein λ chain (249); position homologous to residue 88 of human κ chains, in rabbit anti-DNP γ chains (248); and position 32 in pig anti-DNP λ chains (17)].

A third approach which should prove useful will be to compare sequences of antibodies or of myeloma proteins of known antibody activity; this has already led to useful results, consistent with the findings outlined above. The partial sequences of several κ chains from the anti-γG antibodies of patients suffering from hypergammaglobulinemic purpura have been determined (233). Two such cases were identical, and of κ V-region subgroup I, clearly differing from its basic sequence in positions 13, 14, 24, 31, and 34. The availability of homogeneous antibody preparations (250, 251, 252) and automated sequencing techniques (253, 254) should lead to rapid results in these areas.

It is possible that different V-region subgroups are important in different antibody activities. Thus far there is little evidence for this suggestion from studies of myeloma proteins with antibody activity (255), but Hood et al have reported a selection of κ chain V-region III in rabbit antistreptococcal carbohydrate preparations of restricted electrophoretic and allotypic characteristics (256). Similar antigenic specificities have been demonstrated on some myeloma proteins with similar antibody activities (257, 258), and on different cold agglutinin molecules (259), but it is unlikely that all cold agglutinin κ chains are of the same V-region subgroup (260). Several anti-IgG antibodies from patients with hypergammaglobulinemic purpura had κ chains of different V-region subgroups (233); no strong selection of a particular subgroup was apparent. Thus, selection may occur but is unlikely to be absolute.

On the other hand, a perhaps more surprising phenomenon has occasionally been observed, that of heavy chain C-region selection by different antigens. For example, human antidextran antibodies are preferentially IgG2 (261); human anti-Factor VIII antibodies appear to be preferentially IgG4 (262), and horse antipneumococcal polysaccharide is preferentially a minor subclass (263). These findings are unexpected, since it seems that any heavy chain V-region can associate with any C-region (144, 146). The selection may depend on one or all of a number of factors: the V-region-C-region association may not be random; the distribution of immunoglobulin subclasses in different body tissues may not be random; the triggering of an immune response may depend not only on combination of an immunoglobulin with antigen but on class-specific changes in the constant region of the molecule; the total charge of the immunoglobulin molecule may be important (264); and still other explanations are certainly possible.

The rarity of such antibodies with restricted C-region heterogeneity is in keeping with the apparent well-being of many patients lacking a single immunoglobulin class (IgA) (265, 266) or subclass (267–270). Specific absence of a V-region subgroup has not been described; but in hypogammaglobulinemic patients, the κ chain V-region subgroups were present in different relative amounts (152). The well-described antibody response deficiencies in man (269) and animals (228) are not genetically linked to the known immunoglobulin structural genes. Nevertheless, it is still possible that some immune deficiency diseases will be found to result from deletion or malfunctioning of the structural genes for immunoglobulin V-regions.

CONCLUDING REMARKS

It seems clear that each immunoglobulin chain is the product of (at least) two genes. It is not clear how the integration of these V- and C-region genes is regulated, nor how the product is selected by antigen or other means. Apparently identical V-regions may be associated with different heavy chain C-regions. It appears possible that a single V-region may be expressed on μ chains, and later in the immune response on γ chains. The roles of other immunoglobulin classes in this sequence are not known.

It has been difficult to obtain direct evidence for the number of germ-line genes involved in the immune capability of vertebrates. The "best" theories are therefore those which have good precedents in other biological systems. Recently, multiple DNA sequences which can undergo mutation and evolution at normal rates, and which can apparently evolve by frequent duplication to give patterns of variation similar to those of immunoglobulins, have been discovered. These findings make more likely theories proposing that much immunoglobulin variability is carried in the germ-line, in a large number ($> 10^4$) of genes. The importance of selection of spontaneously occurring somatic mutations during individual development has not been settled, nor has the intriguing problem of why such (multiple ?) genes should apparently segregate in a Mendelian fashion in one case (that of rabbit heavy chains). Techniques other than purely structural studies are now needed to answer these questions.

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PROLIFERATION OF MYELOID CELLS

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INTRODUCTION

The production of granulocytes is one of the most dynamic physiologic activities in animals and man, and involves a relatively large organ, the bone marrow, which weighs 1600 to 3700 g or approximately the weight of the liver (1). Ordinarily, granulocyte production is a well-controlled process with orderly progression from the earliest precursors through maturation and differentiation leading ultimately to release of the polymorphonuclear granulocyte into the peripheral blood. Years ago it was assumed that in leukemia this process was greatly disturbed owing to some unidentified influence, and that the myeloid precursors were rapidly and excessively proliferating without undergoing maturation.

This early popular hypothesis was never substantiated, and it was first suggested by Astaldi & Mauri (2), and subsequently repeatedly confirmed, that leukemic cells are not "wildly proliferating," but that there is some defect which leads to persistence of immaturity and to the accumulation of large numbers of immature myeloid cells (3–6).

The precise nature of this defect has not been elucidated, but the presence of increased myeloid cells in acute or chronic myelocytic leukemia could theoretically result from a number of factors or some combination thereof. These factors include: (*a*) an increase in production of leukemic cells due to an enlarged stem cell or other precursor compartment; (*b*) a marked prolongation of the intravascular survival time; (*c*) a decrease in leukemic cell death and cell loss.

In general, it can be said that under normal circumstances granulocytopoiesis is a cell renewal system such that cell production equals cell death. In patients with leukemia in relapse, granulocytopoiesis exceeds cell loss, resulting in an expanding population. However, in certain circumstances such as in adult acute myelocytic leukemia this may not be the case, and leukemic cell production may equal cell loss so that the peripheral white blood count remains stable for long periods of time.

Granulocytes in the adult animal or man are produced in the bone marrow where, in normal individuals, division and maturation occur from the earliest cell, the stem cell, successively through the various cell types to the mature polymorphonuclear leukocyte so that morphologic compartments are readily identifiable. In the granulocytic leukemias, myeloid production also occurs in the bone marrow but there is evidence from cell kinetic studies and from histological examination that leukemic cells may also be produced in a variety of sites in addition to the bone marrow, i.e., lymph nodes (7), liver, spleen (8), testes, etc.

TECHNIQUES

A great many labels and techniques have been utilized to study myelopoiesis but, unfortunately, there are problems associated with each of them (5). It is beyond the scope of this paper to review every method described in the literature although it is important to mention briefly the major difficulties associated with the techniques utilized most frequently in man.

The techniques now used are fundamentally either histological (mitotic index, for example) or involve the use of radioisotopes. The radioisotopes are of two classes: (*a*) DNA labels, and (*b*) non-DNA labels. DNA labels are those radioisotopically labeled materials which are incorporated into DNA during the phase of the cell cycle when this nucleic acid is synthesized. DNA labels remain with the cell until the end of its life-span and are therefore of value in determinations of rates of production and life-span. The most commonly used label of this type is tritiated thymidine (^{3}H-TdR). Non-DNA labels are those which attach to the cell regardless of the phase of the cell cycle or the stage of maturity. Diisopropylfluorophosphate labeled with radioactive phosphorous (DF^{32}P) is most frequently used although chromium-51 (^{51}Cr)-labeled sodium chromate has recently become popular in the study of leukocyte kinetics (9–11).

Table 1 lists the methods and labels most commonly utilized in the study of myeloid proliferation and kinetics and indicates the major problems associated with each. It is obvious that if some of these obstacles could be overcome the usefulness of many of these techniques would be greatly enhanced.

TABLE 1. METHODS AND PROBLEMS IN THE STUDY OF MYELOPOIESIS AND LEUKOCYTE KINETICS

Method	Problems
I. Radioisotopic Labels	
^{3}H-TdR	Possible damage to genetic material limits its use to individuals with limited life span and nonreproductive. Radioautography and analysis time-consuming and laborious. Expensive. Possibility of reutilization (94).
DF^{32}P	Labels all cellular elements of the blood, hence care must be taken in isolating granulocytes for final specific radioactivity determinations (95). Poor for radioautography.
^{51}Cr	Not a cell-specific label and therefore careful isolation of cells is also required. However, this is useful for body surface scanning, granulocytes must be isolated for labeling prior to transfusion and this makes it difficult to use in individuals (normal or otherwise) with relatively low granulocyte counts.

TABLE 1.—*(Continued)*

Method	Problems
II. Methods	
Mitotic index	Not reliable for measurement of rate of cell proliferation because it is based on incorrect assumption that DNA synthesis plus duration of mitosis are constant. High mitotic count may be due to long duration of mitosis.
Mitotic rate	
Mitotic rate = mitotic time × mitotic count	Requires colchicine or other stathmokinetic agent in vivo.
Mitotic time $= \text{MI} \times \text{Tc}$ (0.693)	Cleaver (96).
Labeling index (^{3}H-TdR) intravenous pulse infusion; local injection (bone marrow).	Limited value because of variation in duration of S. Infusions are very expensive but are used to estimate proportion of resting cells. Assumes that all labeled cells go on to mitosis. Radioautography is time-consuming (see above).
Grain count halving.	Complicated because slightly labeled cells are "lost" and cells from the morphological compartment under study are lost by maturation into next compartment. Radioautography is feasible.
Double-labeling —^{3}H-TdR and ^{14}C-TdR.	Wimber & Quastler (97). Not practical because of expense.
Labeled mitoses (^{3}H-TdR)—determination of cell cycle times.	Second wave of labeled mitoses must be observed to obtain Tc but does not always appear. Provides only an indication of mean generation time.
Cell loss.	Not readily determined with any degree of accuracy in leukopoiesis with present methods. Biochemical marker excretion product) needed.

Abbreviations

^{3}H-TdR—Tritiated thymidine
^{14}C-TdR—Thymidine labeled with carbon-14
DF^{32}P—Diisopropylfluorophosphate labeled with phosphorus-32
^{51}Cr—Sodium chromate labeled with chromium-51
MI—Mitotic index (mitoses per 100 cells)
LI—Labeling index, percent labeled cells
S—DNA synthesis time
Tc—Generation time

STEM CELL AND PROLIFERATING POOLS

Granulocyte production and its control have for a long time been the subjects for a great deal of work and speculation. Nevertheless, relatively little is known about stem cells, particularly in man, although there is a good deal of information which has been derived entirely from animal and tissue culture studies. The stem cell compartment must be able to maintain itself against continued removal of cells for differentiation, to reconstitute itself if depletion occurs, and must be capable of increasing its rate of cell production upon demand. Available evidence suggests that a cell similar to a small lymphocyte may be the common stem cell (12, 13), although a good case can be made for the monocyte (14). In newborn rats that have received continuous ^{3}H-TdR since early conception, all the cells in the bone marrow are initially labeled. However, within six days of birth the label disappears from the identifiable erythroid, myeloid, and megakaryocytic cells but a significant number of cells, not clearly recognizable, remain labeled (15). Presumably, the common stem cell is included in this group.

Studies utilizing the spleen colony technique demonstrate that erythroid, myeloid, and megakaryoctyic cells are probably derived from pluripotential cells (16–18). Bruce & McCulloch (19) suggested that there are two classes of stem cells: colony-forming cells and cells which may be their progeny and which respond to erythropoietin. However, it was subsequently shown that the experimental conditions used, i.e., exogenous and endogenous colonization, probably measure different characteristics of stem cells (20). Additional support for a common stem cell comes from several reports indicating that a heavy demand for one type of cell diminishes the production of the others (21–23). However, cloning experiments using an agar method of tissue culture yield colonies of mixed populations of granulocytic and monocytic cells (24, 25). Upon recloning, these cells can originate from appropriate individual precursors (26). These findings are of great interest but do not rule out the single pluripotential cell theory since it has been shown that the population of cells observed in the spleen colony assay system differs from that in the in vitro culture system (26a, 26b).

In addition to the stem cell compartment which is self-replicating, there is also a large differential proliferating pool consisting of myeloblasts and promyelocytes, which is probably not self-sustaining. There are large and small myelocytes and these two types of cells represent two "subdivisions" of the main pool, the large cells representing a dividing pool supplying cells to the small cell maturation pool (27–29). It is not known whether all of these small myelocytes then go on to divide or to mature and enter the next compartment. A key role has recently been suggested for the myelocytes in which it is believed that granulocytic production is expanded by an increase in the number of myelocyte divisions and a shortening of the generation time (30). This appears to be the most attractive hypothesis at the present and is consistent with most kinetic data.

In assessing normal granulocyte production in the bone marrow the labeling index utilizing ^{3}H-TdR is very useful and provides an estimate of the growth fraction. Since cells in the dividing pools and nondividing pools can readily be detected by morphologic criteria alone, this plus the uptake of ^{3}H-TdR by cells in cycle can be used to estimate granulocyte production. However, in leukemia it is impossible to distinguish between cycling and noncycling blast cells on morphologic grounds alone. It was suggested years ago, based on stathmokinetic and labeling studies, that the proliferative capacity in acute leukemia was very low compared to normal marrow (2, 31). Gavosto et al (6) reported that the in vitro labeling index of blast cells in acute leukemia was in proportion to the size of the cells, but was not related to the phase of the cell cycle. They concluded that the larger cells were the younger ones, and that with aging proliferative capacity was lost.

Mauer & Fisher (32) subsequently suggested that the large leukemic cells comprise a relatively small percentage of the leukemic cells in the marrow (about 12 percent in the two patients reported) with a labeling index, after a single injection of ^{3}H-TdR, of approximately 50 percent. Virtually none of the small cells was labeled. They hypothesized that the large cells were the dividing population but after one or two divisions these cells became smaller and stopped dividing. The small "nondividing" leukemic cells were, however, capable of re-entering the proliferative phase (33). In a patient with acute lymphocytic leukemia following a single injection of ^{3}H-TdR, 10 percent of the small and 75 percent of the large blasts were labeled. By 24 hours, the labeling index of the large blasts had decreased to 22 percent and that of the small cells had increased to 20 percent. During the fourth to the seventh day, nine additional injections of ^{3}H-TdR were given and then 93 percent of the large cells and 72 percent of the small cells were found to be labeled. These results were interpreted as suggesting that the only identifiable source of labeled cells for maintenance of the large blast cell population was the small cell compartment.

Although these studies are quite intriguing, it seems obvious that one of the difficulties with the concept is that the technique ordinarily used to prepare slides usually results in a great deal of variability in cell thickness even on the same slide (34). With the evidence that these small cells may re-enter the proliferating pool, the sharp distinction between these two types of cells becomes rather artificial. The studies of Clarkson are consistent with the suggestion that the vast majority of leukemic cells are capable of DNA synthesis, but that cell cycle times vary greatly (35). Recent studies in this laboratory (36) indicate that at least in one animal leukemia, the small, supposedly nondividing leukemic cells are readily able to induce leukemia and death upon transplantation to normal animals. Regardless of whether one accepts the presence of a so-called nondividing cell population, or a population of cells in G_0, or in a prolonged G_1, it may well be that many of the antitumor agents currently in use do not affect these cells significantly and it is these cells which are of the greatest concern in the treatment of acute leukemia (37). In this connection it is of interest to note that in the steady-state, only a very small proportion of mouse bone marrow cells are in DNA synthesis (38). The greater proliferative state of mouse lymphoma accounts for the marked difference in the sensitivity of these two cell populations to a variety of chemotherapeautic agents in vivo (39).

The observation that a significant proportion of the stem cell population normally is in a resting phase suggests that the output from this pool is probably a function of demand. As indicated above, stem cells in the bone marrow have never been clearly identified and there is considerable controversy as to which cell(s) is the stem cell. In any case, they are probably present in very small numbers; however, Warner & Athens (28) and, more recently, Cronkite (30) consider the myelocyte compartment as the prime source of new cells contributing to the marrow precursor pool. As mentioned above, kinetic data are consistent with this hypothesis. Myelocytes can be divided on the basis of size, into a small and large cell population (40) and it is quite possible that the small cells may be a "nondividing" population. The considerations concerning the G_0 cells may also apply here. It should be mentioned, however, that recently Morley & Stohlman (41), in studying myelopoiesis in mice with shielded marrow receiving X irradiation, found no evidence of a significant increase in the myelocyte proliferative rate when neutropenia developed.

Normally, approximately one-third of the myeloid cells in human bone marrow are in proliferation with an average labeling index of about 30 percent (42). It has been estimated (42) that approximately one cell per 100 normal granulocyte marrow cells is produced per hour and about four cells per 100 myeloblasts are produced per hour. The mean granulocyte production in normal individuals is approximately 1.63×10^9 granulocytes per kilogram of body weight per day (43).

There are good data indicating that granulocyte production is moderately increased in acute and chronic myelocytic leukemia (27, 44–50). Blast generation times in acute leukemia may be within the normal range or longer (27, 48–50) but most of the blasts are either nondividing or in a prolonged G_1 (32, 42). In chronic myelocytic leukemia, generation times are reported to be longer; however, the techniques utilized are of questionable validity (51). In these patients, granulocyte turnover is usually increased above normal but may range from 145×10^9 granulocytes per kilogram per day to as much as 2.0×10^{10} granulocytes per kilogram per day in patients with very high peripheral white blood counts (45). Rough estimates from our own kinetic data in patients with acute myelocytic leukemia reveal granulocyte production in the range of 5×10^8 to 5×10^9 granulocytes per kilogram per day (46, 52).

MATURATION AND RELEASE FROM THE BONE MARROW

Maturation occurs in the nonproliferating pool, consisting of metamyelocytes, band forms, and segmented neutrophils. There is some controversy concerning the most mature cell in animals capable of DNA synthesis and division in the myeloid series, but the evidence in man favors the myelocyte. DNA synthesis does not occur in the nonproliferating pool but other biochemical and morphological changes occur in both nucleus and cytoplasm. The maturation of myeloid cells is morphologically characterized by a progressive condensation of the nucleus and, concomitantly, there are gross changes in the cytoplasm, particularly the appearance of granules. In the mature cell, the nucleus is pyknotic and the granules become quite prominent.

The biochemical events accompanying maturation have not been completely elucidated largely because of technical problems in obtaining adequate numbers of cells at the same level of maturity (53). However, some data are available, obtained chiefly by radioautographic studies and, despite its technical limitations, this approach has yielded important information on the comparative rates of macromolecular synthesis at the various levels of maturation. The rate of DNA synthesis decreases with maturity until the metamyelocyte stage is reached, when it ceases completely. Whether the nucleus of the mature granulocyte can be "reactivated" is unknown. RNA and protein synthesis, like DNA synthesis, also decrease during maturation (54). Similarly, rates of DNA, RNA, and protein synthesis are significantly reduced in leukemic blast cells (55, 54). However, there is a major problem in the interpretation of these data obtained by using labeled precursors, i.e., the variation in the rate of catabolism of nucleosides in cells at different levels of maturity. For example, mature cells catabolize thymidine more rapidly than immature cells (56) which may account for the lower thymidine incorporation into the more mature cells of the myeloid series. Similarly, if a leukemic cell contained more amino acid decarboxylase than its normal counterpart, labeled amino acid incorporation would be reduced and it would be concluded that the rate of protein synthesis was low (53).

However, there are some distinct differences between leukemic cells and their normal counterparts. The deficiency of leukocyte alkaline phosphatase in patients with chronic myelocytic leukemia is well known. A decrease in pyrimidine deoxyribosyl transferase activity in mature granulocytes from these patients has also been described (57). Finally, recent studies of transfer RNA profiles in normal and leukemic lymphoblasts in culture have revealed significant differences in a number of species of transfer RNA (58). These observations have not yet been confirmed but they raise important questions concerning their possible relationship to the failure of leukemic cells to undergo differentiation and maturation. The role of the minor bases of transfer RNA is also of interest and it has been shown that one of these, isopentenyladenosine may both inhibit and stimulate DNA synthesis, transformation, and mitosis of phytohemagglutinin-stimulated lymphocytes (59).

It is of great interest that leukemic cells appear to have the capability of maturing in vitro even after long-term culture. Friend et al (60) reported that murine leukemic cells after propagation in continuous culture for one year were observed to be maturing along erythrocytic lines perhaps due to changes in conditions of culture. Human leukemic myeloblasts may also undergo maturation to segmented neutrophils in culture (61). Murine myeloid leukemic cells in tissue culture can be induced to differentiate in the presence of "inducers" from other normal or leukemic cells (62, 63).

With the completion of maturation, the mature granulocyte enters the so-called "mature granulocyte reserve" of the bone marrow. Estimates vary but there are probably approximately 2–3 × 10^{11} granulocytes in this compartment (29). Approximately 10^7 to 10^8 cells are released into the blood from this reserve per minute under normal circumstances but this rate can be accelerated upon increased demand, i.e., infection and in response to agents such as bacterial endotoxin (64) and etiocholanolone (65). Leukapheresis experiments, particularly in dogs, clearly indicate that the bone marrow reserve is the chief source if not the only one for mature granulocytes in the blood (66, 67). Ordinarily the release of cells from the bone marrow is a function of the utilization rate of granulocytes. At a given moment, the peripheral blood granulocyte count does not reflect the status of the marrow reserve but a persistently elevated neurtrophil count reflects an adequate reserve and an adequate production rate. The marrow reserve affords a huge buffer between production and a demand which may vary greatly and suddenly. Acute depletion of peripheral blood granulocytes such as occurs during leukapheresis results in marked leukopenia since the rate of entry of cells is exceeded but, with the cessation of leukapheresis, granulocytes rapidly enter the blood and the count rises rapidly. During this second phase it becomes very difficult to induce leukopenia since the rate of entry apparently exceeds the rate of removal of granulocytes. Initially, after leukapheresis, there is no obvious evidence in the marrow of an increase in granulocyte production, but within two or three days myeloid hyperplasia becomes quite prominent. When myelopoiesis is prevented with heavy irradiation, leukocytosis in response to intense leukapheresis still occurs.

Leukapheretic studies in man have added little thus far to our understanding of granulocyte production largely because the instruments used do not have the efficiency to achieve adequate depletion of the total blood granulocyte pool (68, 69). Granulocytopenia can be induced but it is usually of short duration. However, the remarkable capacity for myelopoiesis was illustrated in one patient with chronic myelocytic leukemia and an initial white blood count of 76,200 per mm^3 who was subjected to leukapheresis intermittently for nearly one year (69). During that period 1.18 × 10^{13} leukocytes were collected which, if 10^9 cells can be considered to weigh about one gram, would come to 10 kg of tissue. In spite of this, production was able to keep pace with demand and significant leukopenia never occurred. Removal of approximately 5 × 10^{12} cells in a five-week interval resulted only in a decrease of the leukocyte count from 256,000 per mm^3

to 128,000 per mm^3 (70). With the daily removal of 2.0 to 2.0 × 10^{11} cells from patients with chronic myelocytic leukemia, there was a gradual decrease in white blood count which, however, persisted only for a few weeks after the termination of leukapheresis.

There has been a great deal of speculation concerning humoral substances which might be responsible for the control of granulocyte production and/or release. Evidence has been presented for a leukopoietic or leukocytosis-inducing factor which has been obtained from the plasma of animals undergoing repeated leukapheresis or receiving bacterial endotoxin (71), by the study of isolated rat femora and spleens (72, 73). There are problems in the interpretation of this data since it is difficult to separate the observed effects due to the postulated factor from effects which might be due to a granulocyte-mobilizing agent, such as endotoxin or etiocholanolone.

Material with "leukopoietin" activity has been reported (74, 75) from plasma and tissues and from the plasma of dogs recovering from drug-induced myelotoxicity (76). Spleen colonization of shielded femurs in mice markedly increases following irradiation, suggesting that a humoral factor may be responsible (77). Recently it has been reported that sera from AKR mice with spontaneous lymphoid leukemia are capable of stimulating colony formation by mouse bone marrow cells (78, 79). A stimulating factor may be essential to in vitro growth of bone marrow cells (25, 79a). Embryo kidney cells are an excellent source of this material which has been characterized as nondialyzable and thermostable (79a), but mature tissues and well-differentiated cells demonstrate relatively little activity (25, 79a). Mature normal rat granulocytes produce a substance that inhibits the activity of an inducer which is required for the development of granulocyte colonies from normal hematopoietic cells seeded in soft agar (80, 81). There are several recent studies demonstrating that human sera and urine are capable of stimulating the in vitro growth in agar of normal mouse bone marrow cells to form granulocytic and mononuclear colonies (81a, 81b, 81c). Rytömaa & Kiviniemi (82) also proposed that mature granulocytes contain and probably produce this specific feedback inhibitor, a granulocytic chalone. Evidence for an anti-chalone, regulating the rate of granulocyte production has also been presented. The chalone and anti-chalone were partly purified and appear to have molecular weights of 4000 and approximately 3000, respectively. These observations may be similar to those reported a few years ago (83) when it was demonstrated that homogenates of normal and chronic myelocytic leukemic leukocytes inhibited the uptake of ^{3}H-TdR by leukocytes in DNA synthesis. However, incubation supernates from intact leukemic leukocytes enhanced uptake. The inhibitory factor was shown to be thymidine phosphorylase, an enzyme in the "salvage pathway" for thymine nucleotide synthesis and which catalyzes the reversible synthesis and cleavage of thymidine (56). It is heat-labile and nondialyzable, with greater activity in normal and leukemic mature granulocytes than in immature normal and leukemic granulocytes. The physiologic role of this enzyme has not been clearly defined but in cell-free systems thymidine synthesis is favored. The importance of the salvage pathway in the intact animal and in man is not known but in the presence of exogenous thymidine as much as 85 percent of the total DNA thymidine may be derived via this pathway (84). However, even in the absence of exogenous thymidine, this nucleoside may be synthesized from its base and a sugar derived from an appropriate deoxyribosyl source (85) by an enzyme, pyrimidine deoxyribosyltransferase, which is associated with the same protein having thymidine phosphorylase activity (86). In contrast to thymidine phosphorylase activity, which returns to normal or higher levels in leukocytes of patients with leukemia in remission, deoxyribosyltransferase activity remains low in patients with chronic myelocytic leukemia in remission (57, 87).

It is of interest in connection with the foregoing, that Shohet & Gardner (88) have demonstrated that sera obtained from patients with chronic myelocytic leukemia shortly after splenic irradiation inhibited the growth of leukemic cells in tissue culture and suggested that the inhibitory factor might be thymidine phosphorylase. This is only conjecture although it has been postulated that the salvage pathway may be important in regulating DNA synthesis and cell division (53).

The role of the factors mentioned above in myeloproliferative states is also speculative and there are also few data available concerning the control of leukocytosis due to infection or inflammation. Evidence for a granulocyte-mobilizing factor was presented years ago (89) when it was postulated that a polypeptide, "leukotaxine" could be obtained from inflammatory exudates which increased capillary permeability and induced local migration of granulocytes. Confirmatory work has not been reported. Whether chalones, anti-chalones, or similar substances are involved in cyclical neutropenia (90) or in leukemia (91) is unknown. Periodic oscillations of the neutrophils have also been proposed in normal individuals (92). However, analysis of the reported cases indicates that relatively slight alterations in release of cells from the bone marrow or changes in the marginal pool could be responsible and that it might not involve neutrophil production at all.

If a humoral substance is to be considered in the control of myelopoiesis, the concept must explain the increased granulocyte production seen in response to leukapheresis. There have been many hypotheses proposed (93) but there is very little incontrovertible evidence for any of them. However, in view of the leukapheresis experience there must at least be a feedback loop which reflects the concentration of granulocytes in the peripheral blood. In addition, other factors must affect this control mechanism in order to account for the changes in acute and chronic infection, myeloproliferative disorders, infections associated with leukopenia, alterations of white blood count due to bacterial endotoxin, etiocholanolone, extracorporeal irradiation, etc.

It is a curious and unfortunate fact that, in spite of years of work and speculation by many competent investigators, the clear identification and characterization of a humoral substance controlling granulocyte production has not yet been achieved. Similarly, the mechanisms by which granulocytes are released from the bone marrow have not been elucidated. These are obviously extremely significant questions which have important implications in our understanding and perhaps control of myeloproliferative states. Hopefully, solution to some of these problems will be forthcoming in the not too distant future.

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LYMPHOCYTE PHYSIOLOGY[1,2]

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In 1936, Arnold Rich (1) wrote of the lymphocyte, "Ignorance of the function of this cell is one of the most disgraceful gaps in our medical knowledge." Although a remarkable amount of information about the lymphocyte has been obtained in the past two decades, the gap to be filled has turned out to be larger and more complex than Rich might have imagined.

The emergence of the lymphocyte from long obscurity was in large measure due to the work of Ehrich (2) and Chase (3) and their associates, who clearly demonstrated its role in immunological reactions. A major impediment to the understanding of lymphocyte physiology was removed when Nowell, in 1960 (4), discovered that the small lymphocyte was not an end-stage cell as had been previously thought but could proliferate and take on new functions when stimulated appropriately.

The purpose of this review is to survey recent developments in lymphocyte physiology and their relevance to some problems in clinical immunology. It is intended primarily for those whose research or practice has not brought them into direct contact with the field. The space devoted to each topic must be limited, and the authors have chosen to make reference whenever possible to other recently published reviews. Those interested in pursuing various aspects in greater depth may thus more easily appreciate the full scope of the relevant observations. An apology should be made to those whose pioneering work may not have been acknowledged immediately. In most cases, however, the original contributions can be obtained readily from the references cited.

Much information concerning the lymphocyte has been gained from studies on experimental animals. While, in many instances, parallel observations have been made in humans, the relevance of some conclusions may be

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open to question. An attempt has been made to indicate where species differences may be important.

The review is divided into three broad sections: the anatomy of the lymphocyte and lymphoid tissues, the basic physiology of lymphocytes, and their pathology in certain human diseases. Tumors of lymphoid origin and many diseases which affect the lymphocyte secondarily have not been considered specifically.

ANATOMY OF THE LYMPHOID SYSTEM

HISTOLOGY

Within the past few years, it has become apparent that small lymphocytes, although morphologically indistinguishable by conventional microscopy, are, in fact, a heterogeneous population the cells of which have different origins, characteristics, and functions. Attempts have been made by a number of methods to separate the natural mixture of small lymphocytes into functionally distinct populations (5) but these have not yet proved discriminating enough to allow firm conclusions to be drawn. Therefore, in this review when different characteristics of small lymphocytes are described it should be borne in mind that they are not necessarily exclusive. Thus, one "kind" of small lymphocyte may or may not be capable of performing a number of functions.

The small lymphocyte, which is the predominant cell in all lymphoid tissues, is characterized by a scanty cytoplasm which, on electron microscopy, shows a remarkable absence of specific organelles (6). When tritiated thymidine is injected into experimental animals in order to label newly formed cells, the first of the lymphoid series to be labeled are large cells generally referred to as lymphoblasts or simply "blasts." Over the succeeding days, many of these have been shown to mature into small lymphocytes (7). The interpretation of this observation is obscured by the fact that an unknown fraction of them is not brand new, as it were, but has developed from previously differentiated small lymphocytes as a result of natural immunological stimuli.

It is the lymphoblast, or transformed lymphocyte, which gives morphological evidence of the functional diversity seemingly lacking in the small lymphocyte. They range in diameter from 10 to 20 μ and have relatively large leptochromatic nuclei with one or several prominent nucleoli and varying amounts of cytoplasm. Although a wide variety of cytoplasmic organelles may be seen in individual cells, particular attention has been paid to the presence of ribosomal structures because of their role in the production of proteins. These vary in different blast populations from a sparse complement of unaggregated ribosomes to an abundance of polyribosomes and a well-organized endoplasmic reticulum (6, 8). The majority of lymphocytes found in the circulation contain relatively few ribosomes (9) and probably do not synthesize much if any immunoglobulin. In contrast, some lymphocytes in the spleen and lymph nodes, especially in their medullary portions, do contain endoplasmic reticulum and have been shown to produce antibody (10). The major source of humoral antibody is the plasma cell which, when immature, also resembles a lymphoblast (11). The origins and interrelationships of these lymphoid cells are the subject of this review.

The original stem cell for the lymphocyte series is not clearly known but was long thought to be the reticulum cell. The possibility that some lymphocytes can differentiate from reticuloendothelial cells in mature animals is not disproved (12) but in the embryo at least it appears that most come from a bone marrow precursor resembling the hemocytoblast (13). There is evidence that even at this morphologic stage precursor cells can be divided into two populations on the basis of their complement of endoplasmic reticulum (14). Some small lymphocytes which initially have little or no protein synthesizing organelles in their cytoplasm can develop into blasts with many organized ribosomes when subject to an appropriate stimulus (15, 16).

A characteristic morphological feature of lymphocytes of all sizes is a blunt cytoplasmic foot process best seen in motile living cells which gives them a "hand mirror" appearance. Using cinematography, McFarland (17) has demonstrated that this tail-like structure which he has called the uropod is the means by which lymphocytes establish cell-to-cell contact and that it may contain a sensing mechanism by which the cell becomes activated (18).

Cells with the morphologic characteristics of lymphocytes can also develop lysosomal structures and at least some become phagocytic (19), but such cells are probably derived from a distinct monocytic line (20, 21). Histochemical staining has failed to reveal significant amounts of lysosomal enzymes in small lymphocytes. A number of other enzymes including proteases and lipase have been found by various investigators (22, 23).

Development of Lymphoid Tissues

The embryogenesis of lymphoid organs is of interest because of a number of congenital immunological deficiency syndromes in humans which are apparently due to the failure of normal development. Studies of both the ontogenesis and phylogenesis of the system have also been helpful in providing information relating to the interactions of different components of the immunological apparatus.

The first lymphocytes which can be clearly identified as such are found in the embryonal thymus. This observation was made by Beard (24) in 1900, who also noted that lymphocytes apparently began to leave the thymus in large numbers about the time that other lymphoid organs developed. In the human fetus, the first thymus lymphocytes appear at nine weeks of gestation followed at approximately twelve weeks by those in lymph nodes and spleen (25).

Detailed studies by Good (26) and his colleagues have demonstrated that phylogenetically the first species to possess circulating lymphoid cells is the lamprey. Although the lamprey is the most primitive animal which possesses a full complement of immunological responses and in which thymus-like tissue can be identified, some reactivity to foreign substances is present in lower forms. It has been proposed by Burnet (27) that true immunity is characterized by the ability to mount an accentuated or secondary response on re-exposure to a given antigenic stimulus. Such reactivity is temporally correlated with the phylogenic development of antigen-induced lymphocyte proliferation and with the phenomenon of immunological memory. Significantly, these two features are found only in species which possess a thymus (26).

For some time, it was believed that thymus lymphocytes differentiated from the epithelial cells of the third and fourth pharyngeal pouches which form the anlagen of the thymus. More recent studies of the anatomy of the developing organ (28) and of the appearance of cells in the thymus of parabiotic chick embryos (29) have shown that lymphoid cells migrate into the epithelial structure of the thymus from elsewhere. Just prior to the time when the thymus becomes infiltrated by lymphocytes, large basophilic cells resembling hemocytoblasts may be identified in the surrounding mesenchyme. Furthermore, studies of the repopulation of thymus grafts placed into previously thymectomized newborn mice show that the graft becomes repopulated by lymphocytes derived from precursors which originate in the bone marrow (30). It has been suggested that the epithelial cells of the thymus function to stimulate the proliferation of the bone marrow-derived cells (31) and mitoses in the lymphocytes of the thymus cortex do appear to occur in juxtaposition to the epithelial cells (32).

For some time after birth in most species, the thymus is the most active lymphopoietic tissue in the body, thymocytes having mitotic rates five to ten times higher than lymphocytes in the lymph nodes or spleen (33). It has also been observed that in contrast to the spleen and lymph nodes, lymphopoiesis in the thymus is independent of exposure to immunological stimuli.

Several kinds of experiments indicate that the thymus, at least in fetal and early postnatal life, seeds lymphocytes to the lymph nodes and spleen (34–36). The magnitude of this direct contribution is uncertain since those cells subsequently proliferate in the peripheral organs to which they go. In mice, thymus lymphocytes possess a specific "theta" antigen by which they and their progeny can be identified. In the adult CBA mouse, 70 to 80 percent of lymph node cells and 30 to 50 percent of spleen cells carry theta antigen (37) and these are referred to as "T" lymphocytes (38). Experiments in rats involving the use of specific antirat thymocyte antisera have demonstrated that over 75 percent of cells in fetal rat lymphoid tissues possess thymus antigen although it is present in only 7 percent of cells in the adult lymph node or spleen (39). Thus, it seems that in the rat the thymus-derived lymphocyte population decreases with age, or as a consequence of proliferation loses its marker. There are specific areas in both lymph nodes and spleen, the cells of which are dependent upon some activity of the thymus and which become depleted of lymphocytes in thymectomized animals (40). Whether these thymus-dependent populations are all descendants of thymocytes, or could include lymphocytes which come under the influence of the thymus but whose ancestors did not originally pass through it, is not known definitely. The significance of this population of lymphocytes in relation to the immunological function of the thymus will be discussed in a later section.

The thymus contains two populations of lymphocytes—a major one composed of short-lived cells, many or all of which appear never to leave the organ, and a long-lived population, which seems to provide the migrant cells (33). The high rate of cell death which apparently occurs within the thymus is unexplained at the present time.

Studies of various functions of lymphocytes indicate an important role for another organ which, like the thymus, develops in relation to the primitive gut. This structure has been definitely identified only in birds as the bursa of Fabricius, which lies adjacent to the cloaca (26). Extensive investigation in mammals currently points to the presence of an organ which has a function similar to that of the bursa in the bird, but has failed to reveal its exact location. Sites that have been considered include the appendix and the Peyer's patches of the intestine. Extirpation of the entire intestinal tract is not technically feasible in fetal mammals. In young rabbits, removal of the gut-associated lymphoid tissue followed by a lethal dose of radiation and subsequent rescue by the injection of fetal liver cells results in an animal with many of the immunological characteristics of the bursectomized bird (41). In birds, the development of the bursa as a lymphoid organ, as in the case of the thymus, precedes the appearance of lymphocytes in more peripheral sites. However, a role for the bursa comparable to that of the thymus as a source of lymphocytes for peripheral lymphoid tissues is not yet established, and the way in which the bursa exerts its influence is unknown. Lymphocytes which do not seem to be thymus-dependent but which may be under the influence of a "bursa" are currently called "B" lymphocytes.

The peripheral lymphoid organs in adult mammals include the spleen, lymph nodes, and possibly some of the organized lymphoid structures related to the intestine. As previously noted, development of the lymph nodes and spleen in the embryo follows by a few days that of the thymus. Although the reticular framework of lymph nodes is present prior to the ingrowth of blood vessels, the division into cortex and medulla does not occur until vascular connections are established, a fact which may relate to the important role played by the recirculating lymphocyte component (12). The gross organization of these organs is similar, being divided into cortical areas of densely packed small lymphocytes and medullary zones of more loosely distributed cells of a variety of morphological types including large lymphocytes, reticulum cells, and plasma cells. Within the cortex are found the familiar lymphoid follicles which develop as a consequence of immunological stimulation. They are absent, or poorly developed, in animals raised in a germ- and allergen-free environment (2).

Following exposure to antigens, germinal centers appear in the lymphoid follicles (3). Several kinds of evidence which will be discussed later relate the appearance of germinal centers to antibody production, especially 7S IgG antibody. Germinal centers also appear to be the sites of considerable abortive lymphopoiesis, the degenerating cells giving rise to the tingible bodies which are characteristic of well-developed germinal centers (44).

Animals thymectomized at birth show poor development and in some cases a virtual absence of the small lymphocyte population of the inner cortical zones of lymph nodes and of the sheaths of the penicilliary arterioles of the spleen (40). In contrast in most species studied the superficial layers of the cortex and the lymphoid follicles are relatively unaffected by thymectomy. Since bursectomy in birds results in the failure of development of both of the latter areas (26), these observations have led to the designation of separate thymus- and bursa-dependent regions in peripheral lymphoid organs.

Although the zones described above appear to be under the influence of the thymus and a bursa-like organ, the constituent cells in the adult animal do not all derive directly from these two organs. The lymph coming from a resting node contains only a few percent of newly formed lymphocytes, but several days following antigenic stimulation there is a large outpouring of new cells and obvious mitotic activity in the cortex (45). Thus, in the fully developed animal, a major source of new lymphocytes is the peripheral lymphoid tissue itself.

The sparsely populated areas in the lymph nodes and spleen of neonatally thymectomized animals become at least partially reconstituted following the grafting a thymus tissue in cell-impermeable diffusion chambers, an observation which points to a hormonal mechanism (33). If, however, an interval occurs between thymectomy and the replacement of thymus tissue in a diffusion chamber, cytological restoration is not obtained (46). Therefore, in the absence of a thymus the cell population capable of restoring cellularity to thymus-dependent areas seems to atrophy or to become incapable of regeneration. If, at this stage a free thymus epithelial cell graft is made, repopulation follows, apparently from lymphocytes which originate in the bone marrow and which require intimate contact with thymus epithelial cells in order to be capable of establishing a new thymus-"dependent" population (46).

Although lymphopoiesis is extremely active in the thymus, it is apparently not self-sustaining, since thymus grafts are able to supply lymphocytes to peripheral tissues for only a few weeks, after which the graft-derived thymocytes become replaced by cells to host bone marrow origin (47).

It has been estimated that in the mouse, the time required for a bone marrow precursor to pass through the thymus to peripheral lymphoid tissue is 30 to 50 days (47). The bone marrow, therefore, not only supplies lymphocytes directly to peripheral tissues but is a source of thymocyte precursors which may continue into adulthood. Although the direct contribution of cells by the thymus may decrease with age as has been mentioned, even in the adult a functioning thymus is necessary for the restoration of lymph node and spleen cellularity after severe depletion by total body irradiation or antilymphocyte serum treatment (48). Whether, under these circumstances, the reconstituting cells must also actually pass through the thymus or simply come under the influence of thymic hormone has not been determined.

BONE MARROW LYMPHOPOIESIS

The fact that the bone marrow is also a site of vigorous lymphopoiesis was appreciated many years ago by Yoffey (49). Only recently, however, has the magnitude and functional significance of this component been widely recognized. The proliferation of bone marrow cells is unaffected by both thymectomy and extensive lymphadenectomy (50). Lymphopoiesis in bone marrow, as in the thymus, is independent of antigenic stimulation and only under exceptional circumstances shows follicular organization. The generation time of bone marrow lymphocytes is about three days (34). In guinea pigs, it has been estimated that this would provide about 10^9 new lymphocytes per day (34). The majority of these cells are distributed via the blood to peripheral lymphoid organs and appear to populate the "bursa-dependent" cortical areas and the medulla. Bone marrow lymphocytes will not directly substitute for thymocytes in restoring immunity to lethally irradiated thymectomized animals, substantiating the important fact that some functional attribute is confirmed on the stem cells by the thymus (51). This matter will be discussed in more detail in a later section. Whether or not the bone marrow cells serving nonthymus-dependent functions must also be processed by a bursa or some equivalent organ and whether they require an intermediate residence has not been investigated.

The bone marrow also provides lymphocytoid cells which are capable of differentiating into macrophages. It is now felt that these represent a line of cellular differentiation which is distinct from that of the true lymphocyte (52). The relationship between these cells and the macrophages which develop from blood monocytes has not been clarified (53).

LIFE SPAN OF LYMPHOCYTES

One of the important discoveries about the lymphocyte which has been made in recent years is that some of them have extraordinarily long life spans. It should be borne in mind that the term life span may refer either to the time from differentiation to ultimate death of that cell, or to the intermitotic period. In a sense, these are related events since if the total number of cells of a given kind is to remain constant, mitosis must be followed by the eventual death of one of the resulting pair. The problem is complicated by the possibility of morphological or functional differentiation in the absence of DNA synthesis. Although this may occur in the lymphocyte series, it has not been demonstrated directly and the term "life span" is generally used to refer to the intermitotic period.

Based on the belief that thoracic duct lymphocytes were newly formed and on the discovery that the number entering the blood daily from that source was sufficient to replace the circulating population several times per day, it was for many years believed that the life span of lymphocytes was three to four days at most. In 1954, Ottesen (54) determined by radioactive chromium labeling of human lymphocytes that there must be two populations, one with a life span of three to four days and the other constituting the majority of blood lymphocytes with an intermitotic period in excess of 100 days. Subsequently, it has been found that in all species examined there are at least two cell populations, one with a short and the other with a relatively long life span. In the rat, approximately 5 percent of the circulating small lymphocytes have an intermitotic period in excess of 270 days (55). In man, the intermitotic time for some small lymphocytes may exceed 20 years (56, 57).

The composition of various lymphoid tissues with respect to long- and short-lived cells shows considerable species variation. In general, thoracic duct lymphocytes are mostly long-lived, while those in the bone marrow are exclusively short-lived (34). In peripheral lymphoid tissues, many lymphocytes in the thymus-dependent areas are of the long-lived variety while the majority of the cells in the follicles and medulla are short-lived. Blood contains approximately equal numbers of the two populations. The majority of cells found in the thymus itself are short-lived, a paradox which so far has not been explained (34). Since the thymus is originally populated by short-lived bone marrow lymphocytes, it is attractive to speculate that the thymus confers longevity on some of the cells which migrate through it and later become thymus-dependent.

RECIRCULATION

The explanation for the observation that the thoracic duct daily pours into the blood several times the total number of cells present in the blood at any one time was provided by experiments involving thoracic-duct cannulation in rats. These studies performed by Gowans (51) and his associates demonstrated that thoracic duct lymphocytes recirculate from the blood via the postcapillary venules into the cortex of lymph nodes and thence by efferent lymphatics back into the thoracic duct. Furthermore, by means of chronic thoracic-duct drainage, it was possible to deplete the cortical lymphocytes not only from lymph nodes which drain into the thoracic duct but also from those in the distal extremities and from the spleen, indicating that paracortical lymphocytes in all lymph nodes constitute a single recirculating population. The pattern of recirculation within the spleen has been clarified recently (58). The areas within the organ which are most severely depleted by thoracic duct drainage are the periarteriolar sheaths of the cortex, which are also the thymus-dependent zones.

When lymphocytes obtained by means of thoracic-duct drainage are labeled with a radioactive marker and reinfused, the majority of such cells return to the deep cortical areas of the lymph nodes and to the periarteriolar sheaths of the spleen, but not to germinal centers or bone marrow (51). This is additional evidence that the thoracic duct recirculating population is mostly thymus-dependent, a conclusion strengthened by the fact that neonatal thymectomy markedly reduces the number of lymphocytes in the thoracic duct flow (34).

There is a tendency for cortical lymphocytes obtained from a lymph node in one area to home to nodes of the same region when reinfused into a recipient of the same strain. This phenomenon is apparently governed by specific polysaccharides which are present on the cell surface (59). Thus, lymphocytes obtained from the thoracic duct when reinfused go to the abdominal lymph nodes and Peyer's patches. Lymphocytes treated with disaccharidases or neuraminidase lose their ability to seek out lymphoid tissues until they are able to resynthesize their surface polysaccharides (59).

GENERAL PHYSIOLOGY

The lymphoid tissues of the body in the aggregate constitute a major organ system. Precise measurement of the total number of lymphocytes in the body is not possible because they are divided into an unknown number of compartments which probably have widely varying turnover times. Estimates of total cell numbers based on prolonged thoracic duct drainage indicate that in the calf, the total population exceeds 10^{12} cells.

In the human, cannulation of the thoracic duct for periods up to 60 days may remove a total of approximately 3.5×10^{11} lymphocytes (60). Thereafter, the daily outflow of lymphocytes stabilizes at a value of around 0.8×10^{9}. Most of these are large lymphocytes, suggesting that they are newly formed.

Little is known about the factors which control the growth and distribution of the lymphocyte mass. Although the size and number of lymph nodes in humans vary considerably among apparently healthy individuals, there is evidence for factors which govern the body lymphocyte population both regionally and in respect to total numbers. It has been demonstrated that the removal of a few lymph nodes from one area of the body is followed by hypertrophy of the remaining ones (61). There is no question, however, that the most important single determinant of the overall size of lymphoid organs is the degree of immunological stimulation to which they are subjected. Clearly, there must be factors limiting this response. Those involving antigenic stimulation will be reviewed in a later section.

The factors which regulate the blood lymphocyte level are also largely unknown. Lymphocytes which enter the blood are produced by the thymus and marrow at rates which are obscurely controlled. Antigen stimulation results in an outpouring of reactive lymphocytes from the lymphoid tissues, many of which are of "blastoid" morphology (62). This is a transient phenomenon, but in certain diseases such as infectious mononucleosis can result in a marked lymphocytosis. The lymphocytosis which often occurs in pertussis is unique in being composed almost entirely of mature small lymphocytes.

The ability of physiological stress to induce lymphopenia in experimental animals was noted by Rous in 1908 (63). This effect seems to be due to the secretion of adrenocortical hormone which has a profound effect on all lymphoid tissues, including the thymus. Although it is possible that the secretion of adrenocorticoid is a normal regulator of the lymphoid system, the effect of this hormone is a cytolytic one, being mediated through cell respiration (64). This mode of action offers less satisfactory control than one exerted at the level of lymphopoiesis, but the observation that in Addison's disease, or following adrenalectomy in the experimental situation, there is a lymphocytosis and hypertrophy of lymphoid tissue (65) does suggest that baseline levels of steroid secretion could have a controlling function.

A number of investigations has shown that the effect of corticosteroid is primarily on the short-lived lymphocytes. Steroid treatment of experimental animals is followed by the shrinkage of germinal centers and by depletion of thymus and bone marrow lymphocytes (66, 67). The long-lived lymphocytes found in the paracortical regions of lymph nodes and in the thoracic duct are relatively resistant to this hormone.

The administration of thyroid hormone causes a slight increase in the weight of both lymph nodes and spleen, which seems to be dependent on the presence of the thymus (68). Thyroxin has little effect on the weight of the thymus itself. Thyroxin also augments the increase of lymph node and spleen weight after adrenalectomy (69). For the most part, the importance of hormones other than adrenal in the regulation of lymphopoiesis is not felt to be of major physiological importance.

The effect of drugs and other exogenous substances on lymphoid organs has been studied only sporadically. A lymphocytosis with depletion of lymphocytes from the white pulp of lymph nodes and spleen is produced by heparin therapy (70). The blood lymphocytosis is not associated with an increase in lymphocyte output from the thoracic duct, suggesting that the emigration of cells from the blood back into the lymphoid tissues is inhibited, possibly because of the prevention of cells sticking to venular walls which precedes transvascular diapedesis (16). Therapy with diphenylhydantoin sodium (Dilantin) or the related drug 5-ethyl-5-phenylhydantoin (Nirvanol) may induce a lymphocytosis. In humans with aplastic anemia, a transient increase in blood lymphocyte counts has been obtained after therapy with phytohemagglutinin, a glycoprotein obtained from the bean of the plant *Phaseolus vulgaris* (71). Accidental ingestion of the pokeweed berry causes atypical blast-like lymphocytes to increase in the peripheral blood (15). Polymethacrylic acid has been found to produce a marked lymphocytosis (72).

The plasma of patients with some lymphoproliferative disorders contains a lymphocytosis-stimulating factor (73). It is not present in other diseases such as infectious mononucleosis and whooping cough, in which a lymphocytosis also occurs. The factor has also been found in the thymus of young animals but there is as yet no evidence that it has a physiological function.

Little is known about the ultimate fate of the lymphocyte. The problem is complicated by the heterogeneity of these cells. Given an estimated total body population of approximately 10^{11} short-lived lymphocytes in man, there should be approximately 10^7 new lymphocytes produced per minute. In the steady-state one-half this number would have to disappear in the same time and it is strange that their fate should so easily escape detection. Among the many sites which have been suggested as a lymphocyte graveyard is the lumen of the GI tract (23). Lymphocytes are commonly found between the epithelial cells of the gut but the importance of this phenomenon is uncertain. All lymphoid tissues and the accumulations of lymphocytes elsewhere in the body contain scattered nuclear fragments. The finding of tingible bodies in lymph node germinal centers and in the thymus has been previously mentioned. Considering the total volume of lymphatic tissue, we feel that the death of lymphocytes within the lymphoid system at least seems plausible.

IMMUNOLOGICAL ACTIVITIES OF LYMPHOCYTES

The major function of the lymphocyte is to mediate immunological reactions (7). Higher animals have evolved a complex system to deal with the antigenic challenges to which they are subject, and it is becoming apparent that different and distinct populations of lymphocytes are responsible for various components of these responses (74).

The principal cellular events which follow exposure to an antigen may be divided for convenience into those of proliferation and differentiation. Cellular proliferation, in addition to providing the background for differentiation, serves as an amplification process, making possible an intensified response following re-exposure to the same antigen. Furthermore, the ability of lymphocytes to revert from an activated to a resting state, although not fully understood, may be relevant to the phenomenon of recall.

The classical separation of immunity into cellular and humoral components remains useful, although it is now appreciated that cellular events direct the nature and specificity of humoral responses and that immunoglobulin is not the only humoral substance produced as a consequence of these cellular reactions.

ANTIGEN RECOGNITION

Although antigen recognition is a function of the small lymphocyte (75), it is not yet known at what stage in lymphopoiesis and by what means this ability is achieved.

The cytological response in lymphoid tissue to antigen has been widely studied (43, 76, 77). Following the injection of a labeled antigen subcutaneously, it may be traced to the local lymph nodes and found within macrophages in the cortical sinuses and in the medulla (78). Most of this is subsequently metabolized and excreted. A fraction that seems to be important to the progression of immunological events becomes localized on the cytoplasmic processes of dendritic macrophages, which branch extensively between the follicular lymphocytes (78). This localization appears to follow the first evidence of an immune response, since it reflects the synthesis of small amounts of antibody (79) and therefore cannot be the stimulus for the first cytological event.

The possibility that the macrophages which contain most of the antigen serve to process it, and to provide a modified or "super antigen," has been extensively studied by Fishman and recently reviewed (80). It is widely believed, but as yet unproved, that the direct contact of unmodified antigen with sensitive lymphocytes may lead to tolerance rather than immunity (78).

In lymph nodes stimulated by most protein antigens, the first cells to show a morphological response are the small lymphocytes in the paracortical zone, where blastogenic transformation and mitotic activity are seen. Some of these cells later migrate into the medulla and thence into efferent lymphatics. Subsequently, follicular development occurs and germinal centers are formed (42). The cells in the outer or "mantle layer" of the follicles proliferate at a slower rate and are not derived from those in the germinal center (81). Their function is not clear at this time. It is thought that some of them may migrate to the medullary zones and develop into plasma cells, which appear there simultaneously.

Different kinds of immunological stimuli give rise to variations in this basic pattern of cellular proliferation (42). Antigens which produce predominantly cellular immunity or delayed hypersensitivity affect chiefly the paracortical small lymphocytes. When humoral antibody production is the principal consequence, follicular development and the appearance of plasma cells in the medulla are the more prominent events. In the case of polysaccharide antigens, the response may be restricted to the follicular system and medulla (82). Proliferative activity in the germinal centers coincides temporally with the onset of antibody production and antibody is demonstrable in large pyroninophilic cells in the germinal centers (83).

Studies of the cellular traffic in and out of lymph nodes have provided important insights into the mechanism of immunity (45). The efferent lymphatic of a single unstimulated sheep popliteal lymph node carries a steady efflux of about 10,000 cells per cubic millimeter. Only a few percent of these are newly made in the node, almost all the rest belonging to the recirculating long-lived pool. Within minutes after antigenic challenge, the lymphocyte output almost ceases, which suggests that the node may be trapping the recirculating lymphocytes. Of possible relevance is the observation that X irradiation of a lymph node just before antigenic challenge has no effect on its ability to respond to antigen (84). These facts suggest that the antigen-sensitive cells belong to a recirculating population, which becomes arrested in the appropriate node only after antigenic challenge. The immune response of the spleen also depends on its supply of antigen-reactive lymphocytes recirculating via the splenic artery (51).

Several days after primary immunization in the sheep, the number of cells in the efferent lymphatic increases sharply and large numbers of pyroninophilic blast cells appear (45). These cells, some of which specifically bind antigen, are also demonstrable in human peripheral blood after immunization (85). The lymphocytes which appear in the efferent lymphatics of stimulated nodes transfer the ability to respond to antigen to the other nodes in the body (86), and permanent interruption of the efferent lymphatic at this stage prevents systemic sensitization (87).

The destruction of recirculating cells by extracorporeal irradiation of peripheral blood reduces the ability of animals to respond either primarily or secondarily to antigen (88). Studies by Gowans (7) of rats with thoracic duct fistulae showed that animals depleted of recirculating lymphocytes by that method were impaired in their ability to produce a primary but not a secondary response to antigen. Since he also demonstrated that thoracic duct cells from an immune animal could transfer to an unimmunized recipient the ability to give a secondary response on its first contact with the antigen (75), it can be concluded that some memory cells remained fixed in tissues, while others, as well as the immunologically competent but not yet committed lymphocytes, recirculate. Whether the fixed memory cells differ from those which recirculate is not known.

The fact that the first response to most antigens in lymph nodes or spleen is proliferation of the paracortical or periarteriolar sheath lymphocytes suggests that antigen reactive cells are thymus-dependent (42). By using defined populations of syngeneic lymphocytes to reconstitute "lethally" irradiated animals, it has been possible to prove that this is the case at least in some species and for a number of different antigens. Animals, so restored with both thymus- and bone marrow-derived lymphocytes bearing a marker in one population show, in response to immunization, proliferation first of the thymus-derived cells and then of the lymphocytes of bone marrow origin (47, 48). In mice immunized with sheep erythrocytes, Moller & Greaves (89) have shown that the lymphocytes which recognize antigen and transfer memory to irradiated recipients are not directly involved in antibody production but will bind a few erythrocytes to their surfaces to form "rosettes."

The "effector cells" in immunological responses may be regarded as those which produce antibody and the macrophages in cellular immunity. These belong to the bone marrow-derived nonthymus-dependent population (90). It appears that some bone marrow-derived cells also have a specific antigen-recognition mechanism. The functional significance of this dual recognition system is not yet clear. Even prior to immunization with a specific antigen, the bone marrow may contain lymphoid cells which bind antigen

(91), but such binding is not inhibited by the presence of antibody and may be nonspecific. If that is not the case, then it must be concluded that the ability of bone marrow-derived lymphocytes to recognize antigen is not acquired as a result of subsequent lymphocyte interactions but is independently expressed.

It has been widely assumed that the mechanism by which lymphocytes recognize antigen involves the presence on their surface of an immunoglobulin-like molecule. Attempts to demonstrate such immunoglobulin directly, or to passively sensitize lymphocytes with conventional immunoglobulin have been negative or inconclusive. Although a few of the responding cells are able to bind large numbers of antigen molecules, the majority, if they react directly with antigen at all, do so with less than two to three molecules per cell, a smaller amount than is presently demonstrable (92). There is, nevertheless, a growing body of evidence to support the concept that the antigen-recognition site on lymphocytes involves an immunoglobulin-like molecule. Antisera to the light chains of mouse immunoglobulin molecules, when reacted with antigen-sensitive mouse lymphocytes, will block both the binding of labeled antigen and the ability of such cells to give graft-versus-host reactions (93). In contrast, antisera to the heavy chains of serum immunoglobulins both in the human (93, 94) and in the mouse are able to inhibit the antigen-sensing ability of only a small percent of lymphocytes. Although only a minority of lymphocytes bind even anti-L chain antibodies, these observations suggest that antigen recognition could be the result of an immunoglobulin molecule on the lymphocyte surface which possesses an L chain determinant common to humoral antibody molecules but which has a unique or inaccesible heavy chain (95). The situation may be different in the rabbit, where it has been found that antisera to the allotypic markers of both the light and heavy chains of serum immunoglobulin will stimulate a high proportion of lymphocytes to blast transformation (96). This finding is interpreted to mean that the majority of rabbit lymphocytes have membrane-bound immunoglobulin which resembles the circulating immunoglobulin both in its light and heavy chains.

Evidence that the antigen-binding characteristics of the lymphocyte-recognition site are different from those of humoral antibody molecules has been provided by studies of the relative affinities of the lymphocyte and immunoglobulin molecules for synthetic antigens of varying size and complexity. The conclusion drawn from these experiments has been that the receptor on the surface of the lymphocyte is much more discriminating than is the serum immunoglobulin simultaneously produced by the same animal (97, 98). This view has, however, been challenged by those who point out that the discriminating ability of humoral antibody shows a wide spectrum and that some molecules do possess much the same characteristics as the lymphocyte antigen binding site (99). Finally, the fact that memory cells for different antigens can be separated by chromatography, using columns containing the different antigens on particulate carriers (100), is good evidence for the presence of an antibody-like receptor on the lymphocyte surface.

Another problem, at least as controversial as the nature of the antigen receptor site, is to determine at what point in the genesis of individual cells a specific receptor develops. It is believed, based on arguments originally put forth by Burnet (101), that the genetic information necessary to synthesize antigen-binding sites for most if not all antigenic determinants is present in the genome of all cells and that one of these becomes expressed at random in lymphocytic stem cells. Those possessing reactivity against potential antigenic determinants of normal body tissues are then eliminated in the thymus, the rest being released as long-lived lymphocytes into the recirculating pool (101). In support of this proposition is evidence in several species, including man, for a background of antigen-reactive cells in the nonimmunized individual (102). It should be pointed out, however, that the number of such background cells becomes vanishingly small in animals raised germ- and allergen-free. It is very possible that "background" antigen reactive cells develop in response to a previous exposure to cross-reacting antigen (103). A stronger argument for the genomic representation of all antigen receptor sites is the inherited lack of responsiveness to some antigens in certain strains of laboratory animals, which is reflected in a deficiency of the appropriate antigen-reactive cells (104).

A variation of the theory already described holds that lymphocytes, before any contact with antigen, are totipotential (105), but that as a result of an initial contact with antigen in the proper form, they become committed to one reactivity. Evidence in support of this view is necessarily circumstantial, it being impossible by definition to prove that a cell is pluripotential if it ceases to be so after its first stimulus. Studies of the progeny of a single "virgin" lymphocyte for reactivity to more than one determinant are in progress.

The multipotential hypothesis has been tested by attempts to pre-empt the entire uncommitted population of antigen-reactive cells by using a large dose of one antigen followed by another, or by heavily radiolabeling the antigen so that cells reacting with it are destroyed. These studies have given conflicting results. Those involving antigenic competition, although appearing to support the view that there is a single multipotent stem cell pool (106), require a maximum immunological stimulus, and there is evidence that a nonspecific humoral inhibitor of the immune response is made under these circumstances (107). Experiments employing heavily radiolabeled antigens favor the proposition that there are separate precursor cells already committed to a specific antigen (108).

It is unclear whether the existence of immunological memory which expresses itself as an augmented response on re-exposure to antigen is due to an increase in the number of reactive cells, or also to a qualitative change in their function. Not only is the secondary response more rapid and quantitatively greater than the primary, but it also shows in the case of antibody responses a change in the characteristics of the antibody produced, as manifest by its immunoglobulin class and an increased binding affinity for the antigen in question. That a single cell can make only antibody of one specificity is now reasonably well established (109). Siskind & Benacerraf (110) have proposed that the apparent change in the quality of antibody produced represents a natural selection by antigen of those cells whose surface receptors can compete favorably for antigen with the antibody already present in the circulation at any given time. Whether such a process involves the "T" or "B" lymphocyte is not clear but the latter seems more likely.

IMMUNOLOGICAL EXPRESSION—DELAYED HYPERSENSITIVITY

Since it was first demonstrated by Landsteiner & Chase (in 3) that delayed cutaneous hypersensitivity to protein antigens could be passively transferred to an unimmunized animal only by viable lymphocytes and not by humoral antibody, the delayed response has been considered the hallmark of cellular immune reactions. It is becoming increasingly apparent, however, that the cutaneous phenomenon is only one aspect of a larger system of cellular responses which includes cytotoxic reactions, homograft immunity, and the resistance to certain kinds of infection (110a).

Although the recirculating thymus-dependent small lymphocyte is responsible for the initiation of delayed hypersensitivity reactions (51), the cells which are involved as final effectors are a different population which is bone marrow-derived and which accumulates nonspecifically at the reaction site (90, 111). In the cases of delayed cutaneous reactions and resistance to intracellular bacterial infections, the effector cells, although morphologically resembling small lymphocytes when resting, develop macrophage characteristics following activation (52, 111). In some reactions, such as a skin-test site, the local presence of specifically sensitized lymphocytes is probably required (112). The way in which lymphocytes initiate systemic macrophage activation remains uncertain but is thought to involve a humoral factor (113). Whereas peritoneal exudate cells from sensitized mice will transfer immediately detectable skin sensitivity to unimmunized recipients, skin test positivity after the transfer of lymph node cells cannot be detected until after several days (114). Lymph node cell transfers give longer lasting sensitivity than the peritoneal exudate cells, which are mostly macrophages. This experiment, if confirmed, would support the view that two populations of cells are involved in this type of reaction. It is postulated that the macrophages become able to respond to antigen as a result of the passive absorption to their surfaces of an antibody-like receptor made by the lymphocytes (114a). The latent period after lymphocyte transfer is required for host macrophage activation.

The situation is less clear in the case of cytotoxic reactions as studied in tissue culture. In this experimental model, lymphoid cells from a sensitized donor will attach to and kill cultures of nonlymphoid cells derived from a genetically dissimilar strain (115). Lymphocytes, from which the macrophages have been removed, are still able to destroy a histoincompatible cell population (116), and recent evidence indicates that the cytotoxic cells are thymus-derived (117). They appear to be produced by proliferation from a minor fraction of the lymphocyte inoculum (118). Under some conditions, the cytotoxic cells may resemble plasma cells (119), but this seems not to be the case generally (120). There is no evidence to date for the nonspecific recruitment of cytotoxic lymphocytes (121).

The cytological events of homograft rejection have been studied in considerable detail. Following the application of a graft, small lymphocytes in the thymus-dependent areas of the draining lymph nodes enlarge into pyroninophilic blasts and show mitotic activity (122). In some cases sensitization to the graft occurs as a result of the circulation of small lymphocytes through the graft; in others, such as skin grafts, antigen is carried to the local nodes by lymphatics, sensitization occcurring in the absence of blood vascular connections which would allow the circulation of lymphocytes to the graft (123). Origin of the cells which invade and result in destruction of a graft, and whether they are the progeny of thymus-dependent lymphocytes or are bone marrow derived as in the case of lymphocytes in delayed skin reactions, is not yet known. They are characterized by a high mitotic index even prior to the application of a graft (124) which would favor a nonthymus-dependent population. Graft rejection precedes proliferation of the germinal center cells in the local lymph nodes (125).

The lymphoproliferative events which follow homografting are felt to be reflected by the blast transformation and subsequent mitoses which occur in mixed cultures of lymphocytes from two antigenically dissimilar donors (125). This response may be obtained with relatively pure thymus-dependent populations from the thoracic duct but does not show cytotoxic sequelae, perhaps because the necessary effector cells are not present or do not develop.

THE ANTIBODY RESPONSE

Plasma cells are generally acknowledged to be the major producers of circulating antibody. Although the immature plasma cell may be morphologically indistinguishable from a large pyroninophilic lymphocyte, the ability of small lymphocytes to transform into typical plasma cells has not been demonstrated conclusively (126). Antibody-producing cells develop by differentiation as well as proliferation of marrow-derived precursors (127). Cells resembling small lymphocytes in some species can become antibody producers (10, 128) and a very few of the human peripheral blood lymphocytes which may be stimulated in vitro to blast transformation by phytohemagglutinin or specific antigen can be shown by antiglobulin staining to have immunoglobulin on their surfaces (129, 130), but the great majority do not (131, 132). Where a thymus-dependent lymphocyte is required for the initiation of immunological reactions but the antibody-producing cells is of bone marrow origin, it is tempting to postulate some interaction between them. Since, in many cases at least, the antibody-forming cell must also react directly with antigen, the purpose of the two-cell system is not clear. Certain strong antigens are apparently thymus "independent," at least in some species, and can induce a normal response in X-irradiated thymectomized animals reconstituted with marrow cells only (48).

Studies of antibody responses to antigenic determinants (haptens) which require a carrier protein in order to elicit a response have revealed that the responses can be augmented by prior immunization of the animal with the carrier alone (133). Animals made tolerant to the carrier do not produce antihapten antibody when immunized with the complete complex. Current evidence indicates that carrier recognition is a function of thymus-dependent lymphocytes (134). The latter population might function in this situation to make possible antibody responses by "B" lymphocytes to substances having determinants which by themselves are poorly antigenic. The antibody produced under these circumstances has its genetic type directed by the thymus-dependent "T" lymphocytes (135). However, "B" lymphocytes producing different classes of antibody molecules may be induced by a single "T" lymphocyte (136).

HUMORAL MEDIATORS

The search for humoral mediators of cellular immune reactions has proven unexpectedly fruitful. Much investigation has been stimulated by the appreciation that some responses seem to involve cytologic interactions for which humoral factors could be responsible. There is presently evidence of varying strength for the production by lymphocytes of at least nine humoral factors. These are listed in Table 1. Only a few of them have been purified to any extent and it is possible that several of the activities described may be due to a single substance. Some factors are present in normal lymphocytes, while others are made or released following stimulation of previously sensitized cells. The characteristics of these factors cannot be

TABLE 1. HUMORAL FACTORS MADE BY LYMPHOCYTES

Action	Name	Reference No.
A. *Present in sensitized lymphocytes*		
1. Passive transfer—delayed hypersensitivity	Transfer factor	147
B. *Made on contact with antigen*		
2. Attract mononuclear cells	Chemotactic factor	140
3. Inhibit macrophage mobility	Macrophage inhibitory factor	138
4. Enhance killing of phagocytized bacteria	—	113
5. Destroy cells in tissue culture	Lymphotoxin	141
6. Induce contact inhibition in growing cultures	—	145
7. Inhibit viral infection of cells	Interferon	146
8. Induce local inflammation	—	138
9. Induce DNA synthesis in lymphocytes	Blastogenic factor	142

discussed here in detail but have been the subject of a recent symposium (137).

Macrophage-inhibiting factor is released from sensitized lymphocytes following incubation with antigen (138) and is felt to play a role in the accumulation of macrophages at the site of the delayed hypersensitivity reaction. Its production by human lymphocytes can be detected in a system using guinea pig macrophages and this has permitted investigation of human immunological deficiency states involving cellular immunity (139). Chemotactic factor (140) and lymphotoxin (141) may also play a role in delayed hypersensitivity. A skin-reacting factor (138) is produced by lymphocytes from guinea pigs with delayed hypersensitivity following incubation in vitro with antigen. Its relationship to macrophage-inhibitory factor and whether or not it plays a role in the pathogenesis of delayed skin reactions is uncertain (138).

Mitogenic factor (142) may be obtained from immune lymphocytes stimulated by soluble antigens, tumor cells, or allogenic lymphocytes in the mixed lymphocyte reaction. It is capable of inducing DNA synthesis and blast transformation in cultures of nonimmune lymphocytes. Although it may function naturally to augment the immune response by recruiting uncommitted lymphocytes, evidence for such a phenomenon is still fragmentary (143).

The ability of lymphocytes to destroy cultures of cells which differ from them in their histocompatibility antigens or to which they can be attached by certain other measures (115), has been attributed at least in some cases to the elaboration of a lymphotoxin (141). Since, in most cases, tissue damage by lymphocytes requires intimate cell-to-cell contact, it is believed that a humoral mechanism is not always involved (115). Lymphocytes are also capable of inhibiting the growth of allogeneic cells by a process which may not be caused by immunological activation, since it can be demonstrated in thymectomized animals restored with bone marrow cells alone (144). A soluble factor which will inhibit the growth of cells in the tissue culture is made by nonspecifically stimulated lymphocytes (145).

The best-characterized of the lymphocyte factors is Transfer Factor (147). Because its action is related to the induction rather than the expression of immunity, perhaps it should be placed in a separate category. It has been reproducibly demonstrated only in studies on humans. The material which is of low molecular weight is present in immunologically committed lymphocytes and may be released by cell disruption or within a few hours after exposure to antigen. It will transfer cellular immunity to that antigen either locally or systemically to an unimmunized subject. The immunity is long-lasting and so presumably involves the conversion of host lymphocytes to an immunologically reactive state. In support of this fact, Lawrence has shown that lymphocytes which are passively converted themselves contain transfer factor. The mode of action of transfer factor, and whether it is produced by a specific lymphocyte population, is not yet known.

Ribonucleic acid extracted from lymphocytes obtained from immune animals is also able to passively transfer both delayed hypersensitivity and homograft immunity (148, 149). Since it appears that RNA is not spontaneously released by antigenic stimulation, its natural role, if any, is uncertain. Transfer factor is not RNA and its action is not destroyed by incubation with ribonuclease (147).

The soluble effector molecules which are released by stimulated lymphocytes are the subject of intense study. It is anticipated that inhibitors of these factors will also be found. Two have been tentatively identified. One serves to limit the amount of antibody production by lymphoid cells as measured in vitro (107). The other, found in the alpha-globulin fraction of serum, will inhibit the proliferation of lymphocytes in vitro whether induced by specific antigens or nonspecific mitogens (150).

FACTORS CONTROLLING LYMPHOCYTE REACTIVITY—THE THYMUS AND BURSA

It is evident that if immunological responses are to occur in an orderly manner, there must be mechanisms to control the supply of immunologically competent cells, to limit the proliferation of immunologically committed cells, and to prevent the emergence of cells capable of reacting against host antigens. The means by which these controls are exerted is the subject of much investigation.

The influence of the thymus and, inferentially, a bursa system on the size of lymphocyte populations in the peripheral lymphoid organs has been reviewed in an earlier section. The thymus and bursa also appear to control lymphocyte function in a way which may not relate simply to their numbers. Neonatally thymectomized animals have an impaired ability to mount cellular reactions (151). Thymus grafts enclosed in cell-impermeable diffusion chambers can restore cellular immunity to thymectomized newborn animals, which is strong evidence that thymus function may be exerted by way of a hormonal substance. It has now been reported that repeated injections of a thymus extract will also restore immunological reactivity to thymectomized newborn mice (152, 153).

Several unresolved issues cloud the picture of thymus function. Despite the ability of a humoral factor to restore immunological competence after thymectomy, animals so treated remain lymphopenic (154), particularly with respect to the thymus-dependent paracortical areas in the lymph nodes and the periarteriolar sheaths of the spleen. This finding, combined with evidence that the thymus is the site of vigorous lymphopoiesis in young animals, indicates that it may have a function in addition to its hormonal one. There is recent evidence from several laboratories that thymus-derived cells from the initial antigen-reactive population may be further expanded by thymic hormonal action (46, 48).

Although in the adult, thymectomy has little or no discernible effect, if adult animals are severely depleted of their antigen-sensitive cell population, as by total body irradiation, immunological competence cannot be restored by the passive transfer of a stem-cell population in the absence of a thymus (48). In addition, the immunological response to a previously unencountered antigen is impaired if some time is allowed to elapse after thymectomy (48).

The thymus may also play some role in the development of antibody-producing cells. The effect seems to vary in different species and with different antigens. Thymectomy in the newborn rabbit has little or no effect on antibody responses, but it has been shown that neonatal thymectomy in mice inhibits the appearance of antibody-forming cells following primary immunization (33). However, such animals show a secondary response after thymus grafting, indicating that some cellular response had occurred previously (155). Thymectomy in the newborn mouse, in contrast to many other species, is associated with a deficiency of follicular development as well as the cellularity of the paracortical zones of lymph nodes (33). Evidence derived from naturally occurring immunological deficiency syndromes in humans also points to some effect of thymectomy on humoral responses (156). The "helper" role of a thymus-dependent cell in the recognition of carrier protein for antibody responses (138) may be relevant to this problem. Antibody responses in thymectomized mice can be increased to near that of control animals by increasing the dose of antigen by several orders of magnitude (157).

The majority of lymphocytes in the thymus are not immunologically competent (33). Clearly, those lymphocytes which seed the peripheral organs become competent and there is evidence for a minor population of competent cells within the thymus itself (158).

In summary, therefore, the thymus has been shown to be necessary for the maintenance of the full complement of antigen-reactive cells in peripheral lymphoid tissues. Also, in situ processing of newly differentiated antigen-reactive cells may be obligatory for "switching on" the latent reactivity of lymphocytes which come to it from the bone marrow. The clonal selection hypothesis involves the additional proposal that lymphocytes which are potentially reactive against autologous antigen are eliminated within the thymus (101). This activity is a possible explanation for the observed high level of abortive lymphopoiesis within the thymus, but apart from the increased incidence of autoimmune disease in animals and humans with abnormal thymus development, there is no direct evidence in support of the latter thymus function.

It has been proposed by Good and his co-workers that a bursa system exists in the mammal as in the bird, which governs the development of plasma cells and humoral immunity (26). Experiments in chickens have demonstrated quite clearly that bursectomy *in ovo* prevents the antibody response to many antigens and, as previously discussed, development of the follicular lymphoid system in the lymph nodes and spleen. Additional indirect evidence for a separate bursa system is the failure of thymectomy in several mammalian species to interfere with antibody responses; alternatively, bursectomy in birds does not affect the development of thymus-dependent lymphocytes, or the proliferation of these cells in response to antigen (26). Bursa lymphocytes have abundant ribosomes and strongly resemble the plasma cell precursors in germinal centers (11). Supporting evidence for the existence of a bursa system in man is to be found in the study of congenital immunological deficiency syndromes.

It must be mentioned that a striking exception to the roles described for the thymus and bursa is to be found in the fetal sheep. In this animal's intrauterine development, which has been extensively studied by Silverstein and his colleagues (159), an immunological response to some strong antigens appears prior to any apparent development of peripheral lymphoid tissues. Furthermore, neither thymectomy nor removal of the entire gastrointestinal tract at a very early stage in development interferes with immunological responsiveness. Initial studies in rhesus monkeys have substantiated some of these findings (159a). An explanation for this difference between sheep and other mammals which have been studied is not available.

INHIBITION BY ANTIBODY

Other systems controlling the intensity of the immune response have only recently come under investigation. The route and form in which antigen is given, its concentration, and the antibody produced as a result, play a role in governing the cellular immune response (160). Little direct information is available regarding its cellular basis.

It has been known for many years that the passive administration of antibody will inhibit the response to active immunization in an antigen-specific manner. This is the basis of the suppression of naturally occurring rhesus sensitization in pregnancy by the passive administration of rhesus antibody. Current evidence indicates that the persistence of a small amount of antigen probably in or on macrophages is necessary for the continued synthesis of antibody (161). Binding of the antigen by the passively administered antibody is felt by many to be the basis of the inhibitory mechanism. IgG is more effective than IgM, which may be explained by the fact that the higher antigen affinity of 7S antibody molecules allows them to compete more favorably with receptors on cells for the residual potentially stimulating antigen (162). Support for this mechanism is provided by the finding that passively administered antibody is able to inhibit only those cells making antibody which have a lower affinity for antigen (163). Using the response of rabbit lymphoid cells to sheep erythrocytes, it was found that the development of antibody-forming cells was inhibited by antisheep erythrocyte antibody but that blast transformation of the rabbit lymphocytes occurred normally (164). Since the lymphocytes which undergo blast transformation in response to antigen are largely thymus-dependent and not antibody-forming, it appears to be the antibody-forming cell rather than the antigen-reactive cell which is inhibited. A similar result has been obtained in the mouse in which hemolysin-producing cells, but not thymus-dependent rosette formers, are inhibited by the administration of antisheep erythrocyte antibody prior to immunization (165). The fact that light and heavy chains of the synthesized antibody can be separately inhibited by passive administration of the appropriate antibody fragment indicates the inhibitory mechanism involves an effect within the antibody-forming cell (166). These observations provide additional though indirect evidence that the antibody-forming cell also must react directly with antigen. Cellular immune reactions are more resistant to antibody suppression, possibly because "T" lymphocyte antigen recognition differs in its specificity from that of most antibody molecules (160).

THE CYTOLOGY OF TOLERANCE

The study of immunological tolerance has provided considerable information about the cellular basis of the immune response, although the genesis of tolerance itself remains controversial. There appears to be more than a single mechanism for producing a tolerant state (167).

The classical method of inducing tolerance involves the intravenous administration of purified antigen to animals whose immunological responsiveness is limited as a result of either immaturity, drug treatment, or radiation given just prior to the tolerance-inducing injection (168). Following recovery, as judged by normal responsiveness to a second antigen, the animal remains unable to respond to the original antigen when given by a conventional immunizing method. It is possible to make normal animals tolerant if the correct antigen dose and method of administration are chosen. It is evident from such studies that the induction of tolerance bears an intimate relationship to induction of immunity (169) but investigation of the cellular mechanisms involved is just beginning.

The ability to terminate tolerance by the injection of lymph node, spleen, or by thoracic duct lymphocytes from immunized donors, and the failure to transfer tolerance passively to normal animals by means of lymphocytes from tolerant animals, indicates that tolerance is a negative and not a positive state with respect to the specific antigen-reactive cell (168).

Experiments similar to those previously described in which lethally irradiated mice are reconstituted with various marked cell populations indicate that tolerance is dependent on the recirculating antigen-sensitive lymphocytes which in the tolerant animal fail to respond to the antigen in question. Tolerance to sheep cells cannot be broken by infusing tolerant mice with thoracic duct cells from tolerant donors. Interestingly, however, thymus cells themselves even from tolerant animals can restore immunologic competence to previously tolerant syngeneic mice (170), perhaps because the tolerance-producing antigen in this instance does not enter the thymus. The direct injection of antigen into the thymus will produce tolerance (171). The mechanism by which the thymus-dependent population is inhibited from responding to antigen in the tolerant animal remains uncertain. There is evidence that the administration of antigen by routes which allow it to bypass the macrophage-processing step and come into direct contact with antigen-sensitive lymphocytes may inactivate them (168), although other explanations are also available (172).

Tolerant animals fail to show the expected proliferation of thymus-dependent lymphocytes in the paracortical zones of lymph nodes in response to the immunizing stimulus (173) but a small amount of antibody is elaborated. The presence of critical amounts of antibody has recently been implicated in the genesis of tolerance (174). The thymus-dependent, antigen-sensitive cells though specifically blocked are not destroyed because the tolerance disappears spontaneously in time, leaving the animal immunized (168). A different mechanism seems to apply in mice genetically unable to make antibody to a synthetic polypeptide antigen in which it is the antibody-forming, nonthymus-dependent lymphocyte population which is incapable of responding (175). It has been reported also that under certain conditions tolerance in mice to sheep erythrocytes can be broken by bone marrow cells (176). Both populations of lymphocytes may be directly involved, therefore, in the phenomenon of tolerance.

OTHER FUNCTIONS OF LYMPHOCYTES

Prior to the realization that small lymphocytes were responsible for mediating immunological reactivity, many other functions were ascribed to them. The absence of specific organelles in small lymphocytes and the presence of lymphocytes in inflammatory lesions, especially granulomas, led in part to the belief that they performed a trephocytic function, contributing essential metabolites to other proliferating and differentiating cells (177). The frequent finding of "nuclear dust" in such lesions was interpreted as additional evidence for this theory. Although the hypothesis has not been disproven, there is no direct evidence for it.

For close to a century, theories concerning the nature of the hematopoietic stem cell have been vigorously debated. At least in some species stem cells capable of reconstituting the marrow and lymphoid tissues of irradiated animals are present in the peripheral blood (178). The nature of this cell (and more than one kind may be needed) is not known but there is circumstantial evidence that it could be a lymphocyte.

Studies by Yoffey and his co-workers have shown an inverse relationship between bone marrow lymphocytes and other hematopoietic cells under a variety of stimuli (179). The lymphocytes are the first cell type to regenerate in sublethally irradiated mice and their numbers decline as other types appear. Attempts to study the progeny of single cells in the spleen after intravenous injection of bone marrow suspension into irradiated hosts have given conflicting results (105), and no firm conclusions can be drawn at this time. Two populations of stem cells have been tentatively identified in the bone marrow—a basophilic one which is continuously dividing and may give rise to immunologically active lymphocytes, and a pale staining one which synthesizes DNA intermittently and may be hematopoietic (180).

There is little doubt that monocyte and macrophage precursors under certain conditions appear by light microscopy to be indistinguishable from small lymphocytes. The evidence that they are separate cell lines now seems quite convincing, however (52).

LYMPHOCYTES IN DISEASE STATES

The contributions to bedside medicine of studies in basic lymphocyte physiology and immunology are just beginning, but already a number of clinically useful concepts have emerged. Conversely, the investigation of human disorders involving the immune system has contributed to the understanding and interpretation of some experimental observations. Many correlations which have been attempted represent the work of Good and his collaborators.[4]

Diseases involving lymphocytes may be divided into broad categories: lymphocyte deficiency states, heritable disorders of lymphocyte function, tumors of the lymphoid tissues, and other acquired disorders which may involve lymphocytes. Only a few examples of each can be discussed in this review.

IMMUNOLOGICAL DEFICIENCY SYNDROMES

Many parallels can be drawn between the congenital immunological deficiency disorders and studies of lymphoid organ development, both in man and in the experimental animal (Table 2). A complete clinical description of these disorders will not be attempted here but can be found in the appropriate references.

All of the profound immunological deficiency syndromes result in death either in utero or from recurring infections in infancy. The most severe of these, in which there is a total failure of the immunopoietic and hematopoietic systems, has been called reticular dysgenesis. This is presumably owing to a failure of differentiation of the primitive marrow. Infants with this condition are invariably stillborn and lack all hematological elements. Since maternal-fetal blood exchange is known to occur in utero, it is curious why fetuses with this condition are not more often populated by maternal blood cells. In many animals, peripheral blood contains pluripotent stem cells (178). While this has not been directly shown for the human, maternal-fetal transfer has been documented in a child with a lymphopenic immunological deficiency disorder (181). Of course, such a transfer may occur more frequently than is recognized, the children being stillborn because of a graft-versus-host reaction. Only children not inheriting strong dissimilar histocompatibility antigens from the father could be expected to survive.

At the next level of immunological development, there exists a broad spectrum of deficiency syndromes characterized by lymphopenia and poor

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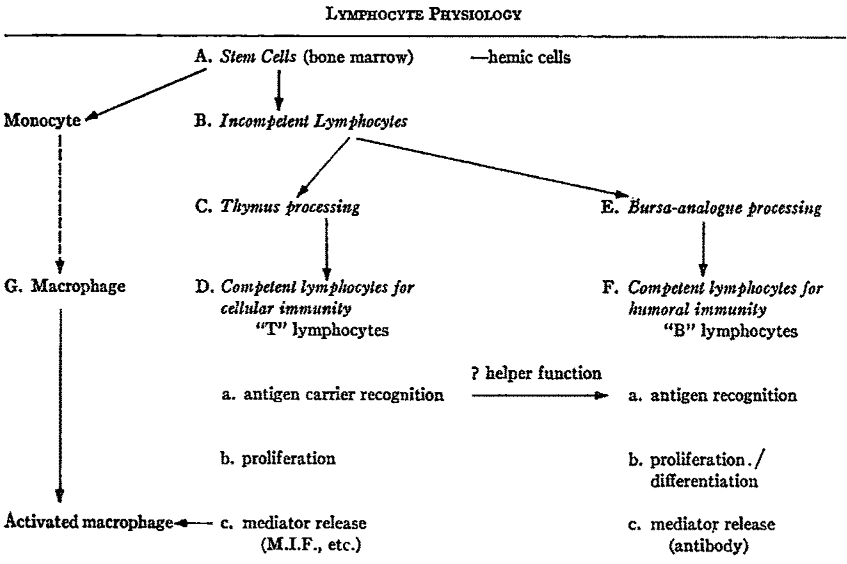
[4] For a review, the reader is referred to a National Foundation Symposium, Vol. 4, No. 1, entitled *Immunologic Deficiency Diseases in Man,* February, 1968.

TABLE 2.


Lymphocyte Physiology
A. *Stem Cells* (bone marrow)
—hemic cells
Monocyte
B. *Incompetent Lymphocytes*
C. *Thymus processing*
E. *Bursa-analogue processing*
G. Macrophage
D. *Competent lymphocytes for cellular immunity*
"T" lymphocytes
F. *Competent lymphocytes for humoral immunity*
"B" lymphocytes
? helper function
a. antigen carrier recognition
a. antigen recognition
b. proliferation
b. proliferation./ differentiation
Activated macrophage
c. mediator release (M.I.F., etc.)
c. mediator release (antibody)

Lymphocyte Deficiency Disorders[a]	
A. Reticular dysgenesis	
B. Lymphopenic (Swiss) agammaglobulinemia Marrow depression (e.g., tumor)	
C. DiGeorge syndrome Nezelof syndrome Thymoma	E. ? Agammaglobulinemia (Bruton type)
D. X-irradiation Antilymphocyte serum therapy	F. Marrow depression or deviation (myeloma)
a. immune deficiency with eczema	a. Wiskott-Aldrich syndrome
b. acquired anergy antimetabolite therapy	b. transient hypogammaglobulinemia of infancy
c. chronic candidiasis	c. dysimmunoglobulinemia ataxia telangiectasia

[a] Adapted from Seligmann, M., Fudenberg, H. H., Good, R. A. 1968. *Am. J. Med.* 45: 817–25.

thymic development (182). In such children, both cellular and humoral immune mechanisms are defective. It is postulated that the developmental abnormality might occur at the stage where bone marrow lymphocyte precursors differentiate. In some of such children, the bone marrow is almost completely deficient in lymphocytes. In the majority, however, some lymphocytes are present even in the peripheral blood (183), and Hoyer et al have suggested that the abnormality might reside in the failure of the normal interaction of lymphocytes with the gut-derived epithelial cells which give rise to the thymus and bursa equivalent (182). The fact that the immunological deficiency of these children can be reversed by normal bone marrow transfusions (184) points to a cellular defect in the precursor lymphocyte. The peripheral lymphoid tissue shows a paucity of lymphocytes in both thymus and "bursa" dependent areas, the lymph nodes and spleen frequently consisting only of reticuloendothelial cells.

In other forms of lymphopenic immunological deficiency syndromes, immunoglobulin levels may be normal, or there is dysgammaglobulinemia (185). The nodes and spleen contain plasma cells and in some cases well-organized lymphoid follicles. The ability to produce antibody in response to specific antigens is usually defective (182). The diversity of genetic defects in cases of lymphopenic deficiency disorders is illustrated by the fact that they occur in both sex-linked and autosomally inherited forms. This, by itself, would indicate the existence of at least several different genotypic abnormalities.

A number of attempts have been made to reconstitute the immunological apparatus of such children with bone marrow and thymus transplants. The danger of graft-versus-host reactions in this undertaking has been well documented. Even peripheral blood transfusions carry a risk (186). Nevertheless, in at least three cases, a successful result with the establishment of stable chimaerism has been reported (184, 187). Various methods have been used to free the marrow infusions of immunocompetent cells which could induce a graft-versus-host reaction. These have included the density gradient centrifugation of the cell population, the use of fetal liver cells which are known to contain an immunopoietic stem cell line (188), and the concomitant use of antilymphocyte serum. In addition to a return of immunological function, it was anticipated that such children should develop normal thymus tissue when stem cells were made available, the thymus epithelial anlage presumably being intact. The appearance of a thymus shadow following bone marrow reconstitution has confirmed this postulate (187). In another child with congenital thymic aplasia, but with an intact lymphoid stem cell population, a successful thymus graft has been reported (189).

Evidence that there may be a bursa-like system of humoral immune responses in man depends strongly upon the existence of another immunological deficiency syndrome, first described in detail by DiGeorge (190). In this condition, there is a failure of development of the structures derived from the third and fourth branchial pouches with the result that children are born without thymus or parathyroid glands. Although most show a failure of cellular immune responses, immunoglobulin levels and humoral immune responses are less impaired. In addition, the peripheral lymphoid tissues show normal follicular and plasma cell development but few lymphocytes in thymus-dependent areas. In some of these patients, antibody production is not normal and germinal center development may be poor also (191). This latter finding is consistent with the observation that neonatal thymectomy in at least some species decreases the proliferative activity of lymphocytes in the germinal centers (33). The explanation for the variable effect on the antibody response of neonatal thymectomy is not clear at this time. An unproven explanation would involve the placental transfer of cells immunologically committed to a few antigens.

In contrast to the syndromes in which a failure of thymus development is felt to be the primary abnormality, there are those characterized by congenital hypogammaglobulinemia but normal cellular immune responses (192). Such children survive virus illnesses normally and can be sensitized to mycobacterial protein. They also reject skin grafts. Serum immunoglobulin levels are low or in some cases undetectable. Fully developed cases have low levels of all classes of immunoglobulin, although there is considerable variation. Serum IgG levels are usually depressed with variable decreases in IgM and IgA. Histologically, the lymphoid tissues are characterized by an absence of the follicular system and by a failure of plasma cells to develop in any location. This constellation is similar to the finding in bursectomized chickens (26). The morphology of the Peyer's patches and other gut-associated lymphoid tissue in such patients is not known in detail. Nevertheless, the existence of these nonlymphopenic agammaglobulinemic syndromes is one of the stronger arguments for the existence in man of a bursa-like system. The fact that in such children normal thymus anatomy is regularly found offers further support for the hypothesis.

HEREDITARY DISORDERS OF LYMPHOCYTE FUNCTION

Within the past few years, some evidence has appeared to suggest that there exists congenital defects in other stages of immunological responses. In these, the cellular development of the lymphoid organs is normal but the production of effector molecules or antibody is deficient. Good and his colleagues have studied children with chronic *Monilia* infection whose lymphocytes, while able to respond in vitro by proliferation on exposure to phytohemagglutinin, do not make macrophage inhibitory factor, and cutaneous reactivity of delayed type is absent (193). A brief report has also appeared of a child with the DiGeorge syndrome, some of whose cellular responses appeared intact in that positive skin responses to mumps and a skin sensitizer (dinitrochlorobenzene) were found as well as normal phytohemagglutinin responsiveness (194). However, the skin responses to some other bacterial and fungal antigens were deficient and no macrophage-inhibiting factor was produced in response to exposure of the child's lymphocytes to *Can-*

dida or streptokinase antigens in vitro (194). An exciting outgrowth of research in lymphocyte physiology has been the recent treatment of children with congenital moniliasis with transfer factor (195). As a result, the treated children have developed cellular immunity against the *Candida* and their lymphocytes have become able to make macrophage inhibitory factor. The administration of transfer factor to a child with Wiskott-Aldrich syndrome has been shown to restore immunological responsiveness (196).

In the Wiskott-Aldrich syndrome, a primary abnormality appears to be the failure of affected children to make antibody to certain polysaccharide antigens (197). Antibody responses to other protein antigens are normal. It has been postulated that the defect may reside in an inability to process polysaccharide antigens which, if so, would suggest defective macrophage function. The restoration of responsiveness by transfer factor indicates, however, that antigen recognition by the lymphocyte may be at fault. An important and so far inadequately explained aspect of this syndrome is the development of deficient cellular immune responses in the later stages of disease. Such children are inordinately susceptible to virus infections and to other intracellular infectious agents, fail to develop delayed hypersensitivity responses when immunized, and show abnormal homograft retention. Lymph nodes which have a normal architecture early in the course of the disease may eventually show a marked depletion of lymphocytes from the thymus-dependent paracortical region. In some, the thymus gland also becomes hypocellular.

A number of other diseases in which chronic infection is present also show atrophy of the thymus and other lymphoid tissues. It is not possible at this time to assert that such changes are a part of the primary pathology. A secondary effect on lymphoid tissues of chronic infection might also explain the deterioration in the lymphoid system of two siblings described by Seeger et al (198), who had chronic respiratory infections and showed progressive lymphopenia and depletion of tissue lymphocytes from both cortex and germinal centers.

Another sex-linked syndrome with an associated immunological deficiency is ataxia-telangiectasia (199). Children with this disorder have a progressive development of telangiectases of the skin of the face and extremities and of the ocular sclerae, with a gradually worsening cerebellar ataxia. Recurrent sinopulmonary infections and impaired cellular immune responses complete the picture. Variable blood and lymph node lymphopenia is found and the thymus is frequently hypoplastic (200). A frequent abnormality found in such children is a low or absent serum IgA or IgE, or both (200). It is possible that a deficient mucosal resistance to infection, especially in the respiratory tract, as a consequence of the immunoglobulin deficiency is the primary defect and that the cellular abnormalities are secondary, as has been discussed above (198). A factor which is capable of inhibiting lymphocyte reactivity has been found in the plasma of children with ataxia-telangiectasia (201).

ACQUIRED IMMUNOLOGICAL DEFICIENCY SYNDROMES

There exists a broad spectrum of cases of hypogammaglobulinemia which is apparently acquired; some of these have been associated with lymphopenia and variable deficiencies of cellular immune responses. Such patients are usually plagued by recurrent infections and have an increased incidence of connective tissue and lymphoproliferative disorders (202). To what degree these disorders involve a primary abnormality of lymphoid tissue is presently a matter of speculation. In a few patients tested, interferon production was found to be normal (203). There is some evidence that dysimmunoglobulinemia may occur in the families of patients affected with acquired agammaglobulinemia with a frequency higher than expected (202), and this would indicate that these syndromes also are due to an inherited abnormality.

OTHER DISORDERS INVOLVING LYMPHOCYTES

The ability of peripheral blood lymphocytes to respond by DNA synthesis to both nonspecific stimuli and specific antigens has been investigated in many diseases. Decreased response have been regularly found in patients with lymphatic leukemia, lymphoma in exacerbation, and sporadically in cases of other malignancies (204–206). Although much evidence points to the importance of cellular immunity as a cancer surveillance mechanism with ability to detect and destroy emerging malignant cells (207), it is not known whether defective cellular immunity in patients with cancer is responsible for the emergence of a tumor or is a consequence of the malignant process (208). The high incidence of tumors, especially of lymphoid origin, in both animals and man with congenital immunological defects, such as the lymphopenic group and ataxia telangiectasia, suggests that any immunological defect may be primary (207). There is also an increased incidence of malignancy in patients with agammaglobulinemia where cellular immune mechanisms are not clearly abnormal (207). It has been suggested that in such cases, a lack of antibody production results in an abnormal chronic stimulus to lymphoid tissues, as a consequence of exposure to many antigens. The development of lymphomas in patients with Dilantin-induced lymphoid hyperplasia (209) is a possible example of such a mechanism.

The role of cellular immunity or a lack of it in other diseases, including the so-called autoimmune diseases, remains controversial. The ability of blood lymphocytes in patients with rheumatoid arthritis (210) and ulcerative colitis (211) to cause cytolytic effects on synovial cells or fetal colonic epithelial cells, respectively, has been reported. Lymphocytes from patients with Guillain-Barré syndrome will respond by blast transformation in tissue culture to an antigen prepared from peripheral nerve (212). The problem of demonstrating abnormal immunological reactivity in disease using the in vitro lymphocyte culture methods is complicated by the finding that in certain phases of illness, a factor inhibiting lymphocyte function may be present in the serum. This fact has been demonstrated elegantly by Turk (213) who, in studies on patients suffering from leprosy, has found that in the lepromatous phase of the disease, serum antibody is present despite a block in the cellular immune response which allows free multiplication of the leprobacilli in the tissues. Treatment which converts patients to the tuberculoid stage is associated with a disappearance of antibody, the return of cellular immunity, and the consequent killing of tissue organisms.

SUMMARY AND CONCLUSIONS

Lymphocytes are the mediators of immunological reactivity. Although lymphocytic stem cells originate in bone marrow, most lymphocytes are produced in the lymphoid tissues in response to antigenic stimulation. They may be divided into several distinct populations, one of which is dependent on the thymus gland for its maintenance and reactivity. Thymus-dependent lymphocytes are primarily responsible for antigen recognition, particularly with respect to its basic molecular structure. They form a pool of cells which are constantly recirculating through lymphoid tissues via lymphatics and the blood. Thus, they become widely available in the body. They also subserve cell-mediated responses such as the activation of macrophages for delayed hypersensitivity reactions.

Another population of lymphocytes which may be under the control of a different gland (bursa equivalent) is the precursor of the antibody-producing cells. Although these also require direct antigenic stimulation, their ability to react with antigen may in many cases involve the cooperation of thymus-dependent lymphocytes. These lymphocyte populations have characteristic anatomical distributions within lymphoid tissues.

A number of diseases characterized by altered immune reactivity may be better understood in the light of recent studies of lymphocyte physiology.

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ABNORMAL HEMOGLOBINS WITH HIGH AND LOW OXYGEN AFFINITY[1]

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The reaction of hemoglobin with oxygen and the mechanisms of adaptation to hypoxia have intrigued scientists for almost a century. Differences in the oxygen equilibria of myoglobin and hemoglobin have been appreciated for about seventy years, and various kinetic interpretations of oxygenation have been presented for nearly half a century. Nevertheless, a deeper understanding of hemoglobin function has only recently emerged, based on the elucidation of the tertiary and quaternary structure of hemoglobin, an appreciation of the allosteric behavior of hemoglobin during oxygenation, and the finding of hemoglobin mutants with altered oxygen dissociation characteristics. These variant structural forms have served as "reagents" in testing hypotheses relating the structure to the function of normal hemoglobin. They have revealed the functional role of specific portions of the molecule and have provided further insight into certain types of human disease. The present review calls particular attention to the effects of hemoglobin variants on oxygen transport.

OXYGEN EQUILIBRIUM

The role of the erythrocyte in oxygen transport is best defined by the oxygen dissociation curve (Fig. 1) in which the saturation of red cell hemoglobin with oxygen is plotted against partial pressure of oxygen. Customarily, oxygen affinity is described as the partial pressure of oxygen at which hemoglobin is half saturated (P_{50}). Under the standard conditions of 37°C temperature and 7.4 pH, the P_{50} of normal blood is 26.6 mm Hg. With an increase in hemoglobin affinity for oxygen, the oxygen dissociation curve is shifted to the left (i.e., the P_{50} falls). With a decrease in hemoglobin affinity for oxygen, the curve is shifted to the right, and the

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TABLE 1. Structural, Functional and Hematologic Data in Hemoglobins Whose Major Functional Abnormality is Increased or Decreased Oxygen Affinity

Variant	Substitution	Importance of Structural Site	Oxygen Affinity	P_{50} mm Hg[a]	Hill's n[a]	Bohr Effect[a]	Hb Concentration[b] (g/100 ml)		Literature Cited
							Males	Females	
Chesapeake	α^{92} Arg→Leu	$\alpha_1\beta_2$ contact	Increased	18–20 (26) B	1.8 (2.9)	Normal	14.5–19.9	14.9–18.0	2
J-Cape Town	α^{92} Arg→Gln	$\alpha_1\beta_2$ contact	Increased	6.5 (8.9) H[c]	1.9 (2.6)	Normal	n.r.[d]	n.r.	3
Yakima	β^{99} Asp→His	$\alpha_1\beta_2$ contact	Increased	12 (26) B	1.1 (2.5–3.0)	Normal	16.5–22.9	15.9–18.2	4, 5
Kempsey	β^{99} Asp→Asn	$\alpha_1\beta_2$ contact	Increased	13.5 (33) H	1.1 (2.7)	Present	17.2–21.3	17.3–20.2	6
Ypsi	β^{99} Asp→Tyr	$\alpha_1\beta_2$ contact	Increased	17.0 (27) B	n.r.	n.r.	16.0–19.0		7 and [e]
Hiroshima	β^{143} His→Asp	DPG binding site	Increased	4.6 (9.2) H	2.0–2.4 (2.8–3.0)	Reduced	—	11.2–17.4	8
Rainier	β^{145} Tyr→Cys	††	Increased	11.0–13 (30–31) B	1.21 (2.66)	Normal	14.9–21.0	16.2–20.8	9, 10, 11
Bethesda	β^{145} Tyr→His	††	Increased	‡‡	‡‡	‡‡	20.5	—	11
E	β^{26} Glu→Lys	$\alpha_1\beta_1$ contact	Decreased	40.5 (30–31) B	2.4 (2.7)	Normal	n.r.	n.r.	12
Seattle	β^{76} Ala→Glu	?	Decreased	41–44 (28) B	2.6 (2.7)	Normal	9.1–9.3	11.3–11.6	13, 14
Kansas	β^{102} Asn→Thr	$\alpha_1\beta_2$ contact	Decreased	70 (25) B	1.1 (2.6)	Normal	13.9	n.r.	15, 16
Yoshizuka	β_{108} Asn→Asp	$\alpha_1\beta_1$ contact	Decreased	9.0 (7.5) H[c]	2.3–3.0 (2.3–2.8)	Reduced	14.7	n.r.	17

[a] Values obtained in blood (B) or unfractionated hemolysates (H). In parentheses values from normal controls.

[b] Values in children included.

[c] Calculated from published oxygen dissociation curves.

[d] Not reported.

[e] D. Rucknagel (personal communication).

†† Substitution probably affects DPG binding and residue $\beta^{98}(\alpha_1\beta_2$ contact). In hemoglobin Rainier a β^{145}–β^{93} disulfide bridge is also formed.

‡‡ Study in progress.

P_{50} rises. The sigmoid shape of the oxygen dissociation curve of normal hemoglobin indicates that affinity for oxygen increases as molecules of oxygen sequentially combine with hemoglobin. This property, which is usually called "heme-heme" interaction, is more properly referred to as "chain-chain" interaction. It is quantitatively expressed by the "n" value in the empirical Hill equation ($Y = kp^n/1 + kp^n$, in which Y is the fractional saturation with oxygen, p is the partial pressure of oxygen, and k is a constant). The n value of normal blood is 2.6–3.0. Absence of chain-chain interaction produces a hyperbolic oxygen dissociation curve with an n value of 1.

The oxygen dissociation curve is subject to modification by certain intracellular ligands, particularly organic phosphates which bind preferentially with deoxyhemoglobin (1). Consequently, the oxygen affinity of the hemoglobin molecule per se can only be determined after preparing a hemolysate from which these ligands have been removed by dialysis or column chromatography. The oxygen affinity of hemoglobin is also strongly influenced by pH. This so-called Bohr effect is determined by relating the change in log P_{50} to the change in pH; the normal value is −0.48. Because of the above-described properties, functional characterization of hemoglobin includes measuring the oxygen dissociation of both intact red cells and purified hemoglobin, calculating Hill's n value, and determining the Bohr effect. As oxygen affinity is dependent on the concentration of hemoglobin, results in hemolysates cannot be compared to those in the intact cell. For an understanding of the physiologic consequences of a mutant hemoglobin, oxygen equilibrium studies in intact cells and in purified hemoglobin fractions are necessary.

HEMOGLOBINS WITH ABNORMAL OXYGEN EQUILIBRIUM

More than 120 abnormal hemoglobins have been described, most of which have normal functional characteristics. Those with abnormal oxygen equilibria can be classed in three categories: (*a*) variants in which the major effect is altered oxygen affinity; (*b*) unstable variants with altered oxygen affinity; (*c*) abnormal methemoglobins. With few exceptions, these variants have been detected in heterozygotes in whose red cells the abnormal fraction is 15–40 percent of the total hemoglobin. The lower proportions are observed in unstable variants.

There are twelve abnormal hemoglobins in which altered oxygen affinity is the most apparent abnormality (Table 1). In eight of these, affinity is increased; in four it is decreased. The hemoglobins with increased oxygen affinity vary considerably in P_{50} and n values. Only a moderate shift to the left occurs in hemoglobins Chesapeake (P_{50} in blood 18–20 mm Hg) and J Capetown; a more marked shift to the left in hemoglobins Rainier (Fig. 1), Yakima (P_{50} in blood 12–13 mm Hg), and Kempsey. When purified, these three hemoglobins display a hyperbolic oxygen dissociation curve with a n value near 1. Both P_{50} and n values of whole blood are lower than would be

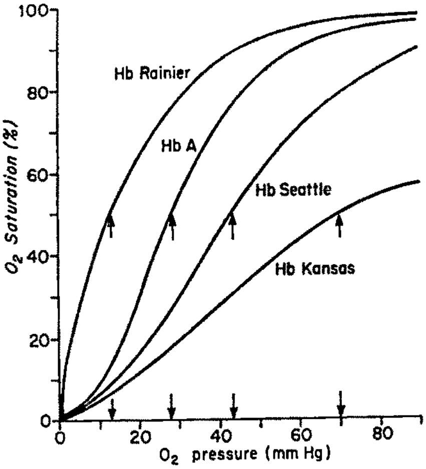

FIGURE 1. Oxygen equilibrium curve of whole blood from subjects with hemoglobins Rainier, Seattle, and Kansas, and from normal controls.

expected from the mixture of normal and abnormal hemoglobin molecules present in the red cell. The possible in vivo formation of hybrid molecules with high oxygen affinity has been offered as an explanation for these low levels (12).

Of the four variants with decreased oxygen affinity, only hemoglobin Seattle (Fig. 1) has been characterized functionally in whole blood, dialyzed hemolysates and purified hemoglobin fractions (P_{50} in whole blood is 41–44 mm Hg). The decreased oxygen affinity of hemoglobin Yoshizuka has only been demonstrated on hemolysates and separated fractions. In hemoglobin E, a significant decrease in oxygen affinity is found only in homozygotes. The n value of these hemoglobins is normal. Very low oxygen affinity (P_{50} in blood is 70–75 mm Hg) together with a hyperbolic oxygen dissociation curve ($n = 1.1$) is characteristic of hemoglobin Kansas (Fig. 1).

It is difficult to study the oxygen equilibrium of unstable hemoglobins. As these variant molecules are susceptible to oxidation and denaturation, only freshly drawn intact cells can be used; there is virtually no information on purified hemoglobin fractions. Included in the category of unstable variants with increased oxygen affinity are hemoglobins Köln (12), Zürich (18), Gunn Hill (19), Freiburg (20), and San Francisco (21); those with decreased oxygen affinity are hemoglobins Hammersmith (22), Torino (22), and Bristol (23).

In the hemoglobins M (variants with methemoglobinemia), oxygen affinity is variably affected (24). Those hemoglobins with alpha chain substitution of the proximal or distal histidine (hemoglobin M Boston, hemoglobin M Iwate) have a decrease in oxygen affinity (25, 26). When there is a beta chain substitution of the proximal or distal histidine (hemoglobin M Hyde Park and hemoglobin M Saskatoon), oxygen affinity is nearly normal (27, 28). Oxygen affinity is reduced in hemoglobin M Milwaukee-1 (29). Most studies of the hemoglobins M have been done on isolated fractions; the oxygen affinities measured are those of the normal chain pair, since the abnormal chains contain ferric iron and are functionless.

The oxygen affinity of purified hemoglobin F is lower than that of hemoglobin A, but red cells containing mainly hemoglobin F have a high affinity for oxygen (see below). One would therefore expect elevated oxygen affinity in adults who synthesize increased amounts of fetal hemoglobin. The oxygen dissociation curve in homozygous hereditary persistence of hemoglobin F is known to be shifted to the left of the normal curve (30); data for homozygous beta thalassemias are not available. Oxygen affinity is consistently decreased (31, 32) in the blood of homozygotes for hemoglobin S and in persons with S-thalassemia (i.e., heterozygous for hemoglobin S and beta thalassemia genes), but oxygen affinity is normal in the blood of heterozygotes for hemoglobin S (32) or in solutions of purified hemoglobin S (33). At the present time it is not known whether the abnormality in oxygen equilibrium in the blood of homozygotes represents a secondary effect of diphosphoglycerate elevation due to anemia and red cell stasis (34) or a direct effect of the molecular lesion.

The Bohr effect appears to be altered in only a few hemoglobin mutants. Reductions have been observed in hemoglobin Yoshizuka (17), hemoglobin Hiroshima (8), two hemoglobins M (25, 26), and purified hemoglobin Rainier (35, 36). The fact that most hemoglobins with abnormal oxygen affinity have a normal Bohr effect is consistent with the reports that the amino acid residues which account for oxygen affinity are different than those responsible for the Bohr effect (37, 38).

MOLECULAR INTERPRETATION OF OXYGEN AFFINITY CHANGES

A model relating hemoglobin structure to function has emerged, mainly from the crystallographic studies of Kendrew, Perutz and colleagues on myoglobin and hemoglobin (39–41). In the hemoglobin tetramer, each chain is a helical compact structure with a hydrophilic surface and a hydrophobic interior in which a heme moiety is attached through several Van der Waals' contacts. By having a noncharged "heme pocket" and an imidazole binding of heme iron, the chain provides an ideal environment for reversible oxygenation. However, in its reaction with oxygen, each individual polypeptide chain of hemoglobin behaves like myoglobin; it has a hyperbolic oxygen dissociation curve (42, 43), unsuited to the requirements of oxygen transport in man.

Compared with the chain, the hemoglobin tetramer represents a higher organization of structure and function. Two alpha chains (α_1 and α_2) and two beta chains (β_1 and β_2) are symmetrically arranged and held together through broad areas of contact, the most important of which is between unlike subunits (α_1 and β_2; α_1 and β_1). Several pieces of evidence indicate that this molecular organization is responsible for the allosteric reaction of hemoglobin (i.e., the enzyme) with oxygen (i.e., the substrate). The sigmoid oxygen dissociation curve is typical for substrate-ligand binding of allosteric enzymes (44). As predicted by the theory of allosteric enzymes (44), hemoglobin has two quarternary structures (41), one for oxyhemoglobin and the other for deoxyhemoglobin. The change from one form to the other is achieved through movement of several amino acid residues (41), located particularly in the contacts between nonidentical subunits. With the older concept of heme-heme interaction, the sigmoid oxygen dissociation curve was attributed to direct interaction between hemes, i.e., the combination of one heme with oxygen caused a change in oxygen affinity of the nonoxygenated hemes. With the present view of hemoglobin as an allosteric enzyme, the chain-chain interactions and the resultant altered conformation bring about the changes in affinity for oxygen. There are, however, ligands which act as allosteric competitors (1, 45). The most important of these ligands is 2,3-diphosphoglycerate (DPG) which combines with deoxyhemoglobin probably at the N-terminal valine and 143 histidine of the two beta chains (46). The result is a shift to the right in the oxygen dissociation curve. Alterations in oxygen equilibrium occurring in hemoglobins with amino acid substitutions directly or indirectly affecting DPG binding, attest to its physiologic importance.

Of those hemoglobin variants with structural abnormalities primarily affecting oxygen affinity (Table 1), nine have substitutions located in areas of the hemoglobin molecule thought to be involved in chain-chain interactions (the $\alpha_1\beta_2$ contact is affected by the substitutions in seven, the $\alpha_1\beta_1$ contact in two). In three of the variants the substitution is at or near one of the DPG binding sites (β143 in hemoglobin Hiroshima and β145 in hemoglobins Rainier and Bethesda). It is possible that in these three hemoglobins, abnormal function is due to conformational changes affecting both chain-chain interaction and DPG binding. Tentative interpretations of the relationship between structural and functional abnormalities have been provided for several of the unstable hemoglobins with altered oxygen affinity (47).

Interpretation at the molecular level is also possible for the increased oxygen affinity of fetal blood. Since the gamma chain residues differ from those of the beta chain at position 143, a difference in DPG binding would be expected. Indeed, the relatively small effect of DPG on the oxygen dissociation curve of fetal hemoglobin has been clearly demonstrated (46). The increased oxygen affinity of blood containing fetal hemoglobin can thus be attributed to decreased binding of DPG.

Physiologic Implications

The sigmoid shape of the oxygen dissociation curve is particularly suited to fulfill its physiologic role. At alveolar oxygen tensions (pO_2 90–100 mm Hg) the curve is flat; therefore a fall of 25–30 mm Hg in alveolar O_2 ten-

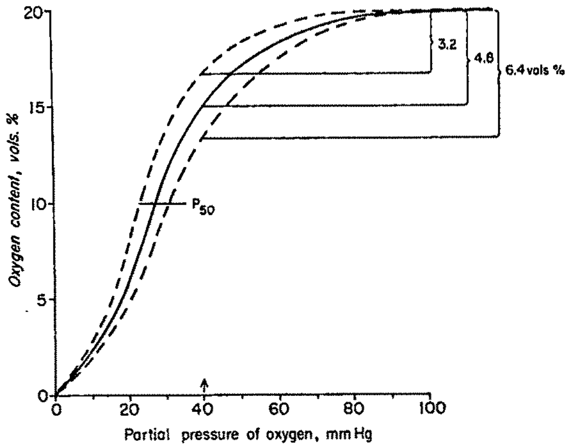

FIGURE 2. The normal oxygen dissociation curve (P_{50} 26 mm Hg) compared with the oxygen dissociation curve of a blood sample with an increase in oxygen affinity (P_{50} 22 mm Hg) and a blood sample with decreased oxygen affinity (P_{50} 30 mm Hg). Note that a 4-mm Hg decrease in P_{50} (curve shifted to the left) reduces oxygen availability by 1.6 volumes percent, whereas a 4-mm Hg increase in P_{50} (curve shifted to the right) increases oxygen availability by a similar magnitude.

sion must take place before there is a significant reduction in O_2 uptake by the blood. On the other hand, small changes in venous O_2 tension markedly alter oxygen release because of the steep slope of the dissociation curve at this range of oxygen tensions (Fig. 2). Thus, a shift in the oxygen dissociation curve usually has little effect on blood O_2 uptake, but it has a considerable effect on the delivery of O_2 to tissues. An increase in oxygen affinity of 4.0 mm Hg decreases O_2 delivery by 1.6 volumes percent, whereas a decrease in O_2 affinity of similar magnitude is associated with a rise in O_2 delivery of 1.6 volumes percent (Fig. 2). If oxygen consumption, cardiac output, and hemoglobin concentration remain unchanged, the effect of an abnormal hemoglobin with altered oxygen affinity would be to modify the venous or tissue oxygen tension. This defect provides a unique opportunity to study adaptive processes.

In normal man, factors involved in oxygen transport include pulmonary function, cardiac output, hemoglobin concentration, and hemoglobin oxygen affinity; these are in equilibrium to maintain normal tissue oxygen tension under varying oxygen requirements. When a single component within this system is altered, compensatory changes occur. Factors within the red cell environment such as DPG and pH may change in a manner to augment oxygen release (48–52). Cardiac output may increase, and those circumstances in which oxygen loading of the blood is incomplete may be improved by increased ventilation. Often, as with anemia, a combination of factors is required, involving changes in both circulation and oxygen release. With hemoglobin variants having altered oxygen affinity, there appears to be one primary compensation: a change in the hemoglobin concentration of the circulating blood. Thus, a hemoglobin with increased oxygen affinity (decreased oxygen availability) is associated with an elevated hemoglobin concentration; a hemoglobin with a decreased oxygen affinity (increased oxygen availability) is associated with a lowering in circulating hemoglobin level. The mechanism by which these changes occur has been clarified by examining erythropoietic regulation.

Hemoglobin concentration in the normal individual is known to be controlled by the output of erythropoietin, predominantly from the kidney (53). Any change in hemoglobin concentration causes a reciprocal change in erythropoietin, as measured in plasma or urine by bioassay techniques. The renal erythropoietin-producing center appears to be relatively insensitive to blood flow changes, as judged by the negligible response to decreased renal blood flow in cardiac failure. The departure from normal hemoglobin concentration in individuals who have an abnormal hemoglobin with altered affinity for oxygen suggests that both hemoglobin concentration and affinity for oxygen are of importance in the regulation of erythropoietin production. Detailed studies of patients with hemoglobins Seattle (13), Rainier and Yakima (10) show that erythropoietin levels were normal despite marked differences in circulating hemoglobin concentrations. Furthermore, when the elevated hemoglobin concentrations of patients with hemoglobins Rainier and Yakima were reduced to normal by phlebotomy (10), the amount of erythropoietin and consequent erythrocyte production increased above that in the normal subject with corresponding hematocrit levels. This finding indicated that erythropoietin response depended on available oxygen and presumably on tissue oxygen tension.

The first effect of hemoglobins with abnormal oxygen affinity is therefore to change the amount of oxygen available to the tissues at any given oxygen tension. Tissue oxygen tension is decreased at a normal level of hemoglobin concentration when the abnormal hemoglobin has increased affinity for oxygen; conversely, tissue oxygen tension is increased at a normal hemoglobin concentration when the variant hemoglobin has a decreased affinity for oxygen. The erythropoietin system responds to these altered tissue oxygen tensions by changing red cell production until a normal tissue oxygen tension is reached. Thus, the hematocrit of 30 in a patient with hemoglobin Seattle and the hematocrit of 60 in patients with hemoglobins Rainier and Yakima represent compensations in red cell mass engineered to normalize tissue oxygen tension. The normal erythropoietin levels in these patients reflect the successful compensation.

The above concepts concerning erythropoietin regulation also apply to the more complicated situations where a hemoglobin variant is associated with both oxygen affinity changes and hemolysis. While it is reasonable to assume that hemolysis always produces some degree of anemia, destruction rates do not explain the wide variations in hemoglobin concentrations observed. Indeed, in patients who have unstable hemoglobins with increased oxygen affinity (Köln, 54–56; Zürich, 57, 58; San Francisco, 21; and St. Mary's, 59), there is a normal hemoglobin concentration in spite of active hemolysis; hemoglobin San Francisco is even associated with mild polycythemia (21). The rate of hemolysis is no greater in patients whose unstable hemoglobin has decreased affinity for oxygen (Hammersmith, 60, 61; and Bristol, 23); nevertheless, they have hemoglobin concentrations of 6–8 g percent. Further evidence that the change in affinity is the major factor in setting the level of hemoglobin is provided by the good correlation observed between hemoglobin concentration and red cell P_{50} over a range of P_{50} from 21–48 mm Hg (62).

There are two hemoglobins which, on first examination, appear not to fit the above concepts of compensation: hemoglobins Kansas and H. Hemoglobin Kansas has a low oxygen affinity but is not associated with anemia (15). In this instance, the oxygen affinity is so low that the arterial blood is only 70–75 percent saturated at normal oxygen tensions. Because of this arterial oxygen desaturation, the normal hemoglobin concentration is required to maintain oxygen delivery and normal tissue oxygen tensions. In double heterozygous alpha thalassemia (hemoglobin H disease), there is hemolytic anemia and the presence in red cells of hemoglobin H (proportion 5–35 percent of total hemoglobin). Hemoglobin H has a high oxygen affinity (42); yet, in the red cells of patients with hemoglobin H disease, the independent reaction with oxygen of hemoglobins A and H results in a biphasic oxygen dissociation curve which is normal at the physiologic range of oxygen tensions (12). Hence, in hemoglobin H disease, there is no reduction in oxygen availability as seen in the other hemoglobins with high affinity for oxygen. Here, there is poor compensation of the hemolytic process, but this is mainly due to a failure in red blood cell production (ineffective erythropoiesis).

Interpreting the physiologic implications of an abnormality of the Bohr effect can only be speculative at present. During exercise, there is normally a pH-mediated shift in the oxygen dissociation curve to the right, which improves oxygen delivery. In hemoglobins with reduced or absent Bohr effect, pH changes during exercise would not be accompanied by this shift and an impairment in oxygen delivery would occur.

CLINICAL ASPECTS

With the exception of hemoglobin E, all of the hemoglobins with abnormal oxygen equilibria are rare. Even with hemoglobin E, oxygen affinity changes are infrequent since they are found only in homozygotes; in the other rarer variants affinity changes occur in heterozygotes; consequently, the physiologic effects of these rare hemoglobins recur in successive generations and a pedigree analysis is useful in making a diagnosis.

In all but one instance, stable hemoglobins with increased oxygen affinity have been detected because of their association with polycythemia. In a few cases, the original diagnosis was polycythemia vera, and patients were treated by phlebotomy or ^{32}P. In all the reported pedigrees, the sole hematologic abnormality was an increase in hemoglobin concentration, with an increase in red cell mass, when this measurement was made (4, 6, 10). Platelet and white cell counts were within normal limits. In no instance was splenomegaly detected. The degree of hemoglobin elevation could be correlated with the extent of the shift in oxygen dissociation curve.

In patients having a hemoglobin variant with a low oxygen affinity, the clinical manifestation is anemia. However, the anemia is a laboratory rather than a physiologic deficit, since it represents a compensation designed to normalize oxygen transport.

When a hemoglobin molecule with abnormal oxygen affinity is also unstable, the cardinal manifestation is hemolysis. Since compensation for the hemolytic process depends on the oxygen affinity of the abnormal hemoglobin fraction, clinical management includes an assessment of oxygen supply rather than hemoglobin concentration per se. In patients with one of the hemoglobins M, the presenting sign is cyanosis. Since half of the hemoglobin M fraction is functionless for oxygen transport, these patients have less oxygen-carrying capacity than their hemoglobin concentrations suggest.

As suggested in the preceding section, the presence of a stable hemoglobin variant with altered oxygen affinity is unlikely to be accompanied by clinical symptoms because of the effectiveness of compensatory mechanisms. Theoretically, one might expect that abnormal relationships between the oxygen dissociation curve of a mother and her fetus might result in asphyxia and death of the latter; however, this predicted difficulty is not supported by existing information (63). In several instances the maternal oxygen dissociation curve was shifted to the left of the fetal curve (e.g., mother with hemoglobin Rainier, blood $P_{50} = 12$ mm Hg; fetus, theoretical blood $P_{50} = 20$ mm Hg); nevertheless, fetal development proceeded along normal lines. In adults with hemoglobin variants and compensatory changes in hemoglobin concentration, the limitations in oxygen transport might emerge under maximal stress. Thus, when the hemoglobin has decreased oxygen affinity, the presence of a reduced red cell mass could resemble bona fide anemia in that there is a reduction in total oxygen available under maximal exercise. Similarly, when the aberrant hemoglobin has increased oxygen affinity, one might anticipate that the resultant increase in red cell mass could induce a viscosity load sufficient to interfere with maximal blood circulation rates. However, it has been difficult to elicit definite exertional symptoms in patients with altered hemoglobin concentration, and at present little can be said about the limitation in performance except that it is minimal.

LABORATORY ASPECTS

In the past, detection of abnormal hemoglobins has been largely dependent on observing fast or slow electrophoretic migration. Since no more than half of the possible amino acid substitutions in the hemoglobin chains would be expected to cause a difference in electrical charge, many hemoglobin mutants cannot be recognized by electrophoresis. Of the eight hemoglobins with increased oxygen affinity which were detected because of an increase in the hemoglobin concentration of the carriers, two (hemoglobins Rainier and Bethesda) had no detectable abnormality when tested in starch gel electrophoresis (10, 11). The unstable hemoglobins are usually detected because the propositus has chronic nonspherocytic hemolytic anemia; in several (64) the electrophoretic migration is normal. Therefore, when mutation produces a disturbance of hemoglobin function, initial suspicion of the hemoglobinopathy is mainly based on clinical considerations. Laboratory confirmation is based on the use of techniques which uncover the molecular functional abnormality. Thus, in diagnosing the unstable hemoglobin variants, tests of principal importance are those which demonstrate molecular lability (Heinz body and thermal stability tests). Similarly, the laboratory diagnosis of polycythemia or anemia caused by hemoglobins with abnormal oxygen affinity is based on the study of the hemoglobin-oxygen equilibrium. As noted earlier, the oxygen dissociation curve must be measured on whole blood, on dialyzed hemolysates (stripped of 2,3-DPG) and on the chromatographically purified abnormal hemoglobin. These procedures are particularly important for the recognition of hemoglobins with decreased oxygen affinity, since anemia per se (i.e., unassociated with hemoglobin structural variation) is associated with a compensatory increase of red cell DPG (51, 65–67) and, consequently, with a shift of the oxygen dissociation curve to the right. In certain deficiencies of glycolytic pathway enzymes (e.g., pyruvate kinase deficiency), the increase in DPG is such as to increase P_{50} values in the blood of patients by almost 10 mm Hg (68, 69). Young erythrocytes, as found in any hemolytic anemia or after hemorrhage, have a decreased hemoglobin affinity for oxygen because of an increased DPG concentration (70, 71). Thus, detection of a decreased oxygen affinity in the blood of an anemic patient does not necessarily make the diagnosis of a functionally abnormal hemoglobin. The procedures required for such a diagnosis are outlined in the paper describing hemoglobin Seattle (13).

CLINICAL MANAGEMENT

In patients who have hemoglobins with abnormal oxygen affinity, therapeutic considerations are more properly related to what not to do, rather than to a positive course of action. For example, a moderate degree of anemia in the presence of a decreased oxygen affinity is not abnormal in a physiologic sense and therapeutic measures such as blood transfusions are inappropriate. Similarly, polycythemia compensatory to the presence of a hemoglobin with an increased oxygen affinity should probably not be modified by phlebotomy or marrow suppressants since oxygen transport would be unfavorably affected. There is little evidence to link the mild polycythemia which occurs in response to hypoxia with cardiovascular complications. Although the frequency is low, patients with these hemoglobin abnormalities have produced a major change in our thinking about oxygen transport in polycythemia and anemia. It is apparent that the efficiency of oxygen supply has correlated with hemoglobin concentration only if all other factors of the oxygen transport system remain at their "normal" levels. Therapy aimed at altering the hemoglobin concentration per se may remove a valuable compensatory change required by the body.

CONCLUSIONS

Hemoglobins with altered oxygen affinity are variants in which the structural abnormalities affect the normal function of hemoglobin as an allosteric enzyme. They are distinguishable as variants associated with increased or decreased oxygen affinity; they may be stable or unstable. In patients whose abnormal hemoglobin has increased oxygen affinity, the consequent reduction in available oxygen is compensated by an increase in red cell mass. Thus, when the hemoglobin variant is stable, the clinical consequence is polycythemia. When the molecule is unstable, the patient's hemoglobin concentration is higher than expected from the severity of the hemolytic process. In patients whose abnormal hemoglobin has decreased oxygen affinity, there is enhanced tissue oxygen supply and reduced bone marrow response. If this variant molecule is stable or only mildly unstable, the consequence is a reduction in hemoglobin concentration without anemia in the physiologic sense; if the variant is unstable, hemoglobin levels are reduced below that expected from the degree of hemolysis. Diagnosis of these variants requires the application of techniques for measuring hemoglobin-oxygen equilibrium in blood, hemolysates and isolated hemoglobin fractions.

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INDUCIBLE METABOLIC ABNORMALITIES DURING DEVELOPMENT OF OBESITY[1]

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"Obesity is a disorder which, like venereal disease, is blamed upon the patient."—E. B. Astwood (1)

INTRODUCTION

The attitude reflected in the above quotation from Dr. Astwood's classic address in 1962 on the "Heritage of Corpulence" has been the prevalent one for some years. Except in rare instances of hypothalamic injury or Cushing's syndrome, the obvious positive caloric balance of the obese is blamed upon the patient and the disorder is labeled "exogenous" obesity. The overweight today are discriminated against and physicians project on them their frustration in usually not being able to effect a cure. Over recent years, however, three types of evidence have accumulated which have lead to a suspicion that there may after all be formidable inherited anatomical or biochemical defects which, together with the factors in our society which promote overnutrition, lead to obesity in certain individuals. First, there is the evidence that at least some obese patients from an early age are burdened with many more than the usual quota of adipose (and other) cells. Second, when more sophisticated techniques, such as radioimmunoassay of peptide hormones, were applied to the study of biochemical changes in obesity, it was found that there were deviations from the normal range, especially in the striking hyperinsulinemia. Some of the deviations are itemized in the left-hand column of Table 1. Finally, an old and oft-refuted doctrine that the body could adapt to excess caloric intake by altering its energy metabolism, the "Luxuskonsumption" of the early German writers, has again been brought under serious consideration. It thus becomes more difficult to attribute obesity to a simple behavioral disorder. The obvious question arises, however, as to whether the derangements observed are the consequences of the obesity or part of the cause.

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TABLE 1. ENDOCRINE AND METABOLIC CHANGES IN SPONTANEOUS AND EXPERIMENTAL OBESITY

	Spontaneous obesity	Ref. no.	Experimental obesity	Ref. no.
Adipose tissue				
Cell size	↑	59, 60	↑	11
Cell number	↑	18	N	
Sensitivity to insulin in vitro	↓	19	↓	10
Sensitivity to insulin of forearm in vivo	↓		↓	
Heart and viscera				
Cell number	↑	12		
Cell size	↑			
Muscle, forearm metabolism				
Response of glucose uptake to insulin	↓	19, 23	↓	10
Response of amino acid to insulin			↓	unpub
Blood lipids			Normal	
Cholesterol	↑	9	↑ (n. limits)	8, 9
Triglycerides	↑		↑ (n. limits)	
Free fatty acids	N or ↑	61	↓ (n. limits)	24, 26
Glucose tolerance				
Oral	N or ↓	9, 23	↓ (n. limits)	8
Intravenous	N or ↓		↓ (n. limits)	
Plasma insulin				
Fasting	N or ↑	23–26	N or ↑	8
Stimulation by glucose	N or ↑	22, 26	N or ↑	
Nonsuppressible insulin-like activity	↑	21	↓	
Plasma glucagon				
Fasting	↑	35		
Suppression by glucose	+			
Plasma growth hormone				
Response to glucose	↓	23, 38–39 42–44, 62	↓	8
Response to arginine	↓	23, 62	↓	unpub
Nyctohemeral secretion		9	↓	9
Adrenal corticosteroids				
Plasma cortisol total	↓	9	N or ↓	8, 9
Plasma cortisol free			N or	8
Total 17-hydroxy	↑ N/kg		N/kg ↑	8
Behavioral				
Spontaneous activity	↓	46	↓	8
Appetite late in day	↑		↑	8
Spontaneous return to initial weight	+		+	
Calories required to maintain obese state	Approx 1300/sq. M	58	Approx 2700/sq. M	unpub

N = within normal limits (see text).

unpub. = unpublished observations of the Vermont study.

Reference numbers in some instances refer to sources from which the specific reference may be obtained.

To attempt to answer this question it seemed logical to study pure exogenous obesity in volunteers with no family history of diabetes or obesity who were willing to gain weight by overeating. There is precedence for this in the study of body composition following weight gain by Keys (2) and in that of the effect of overfeeding on plasma lipids by Mann (3). Other short-term studies of overfeeding have also been done (4–7). We have been engaged in such a study, first with the collaboration of amateur student weight-gainers at the University of Vermont, and later with successive groups of dedicated professional weight-gainers at the Vermont State Prison. For those who may be properly concerned regarding the justification for such a study, we can report that to date all have lost weight spontaneously to basal weight and there have been no evident adverse effects. The project has been designed to provide a constructive group activity for the young institutionalized men and has yielded dividends in education and in rehabilitation. The initial findings of this study are reported elsewhere (8–11) and are summarized in the right-hand column of Table 1. Many of the changes found in experimental obesity were the same or in the same direction as those in spontaneous obesity. However, it is not justified to conclude, as some have done in citing this work, that since the changes in spontaneous obesity are also found in experimental obesity, the same abnormalities are necessarily secondary. All that has been shown is that they may be secondary. If, in an affluent society, there is such an entity as pure exogenous obesity as a result of overeating combined with diminished activity, or from psychological stress, the changes to be expected should be those found in experimental obesity. But far from giving readily interpretable answers to the questions of which factors may be primary or secondary in spontaneous obesity, the results to date have raised many new questions.

In the present review, we will consider the various parameters studied in the light of three main questions: 1. Does the production of experimental obesity lead to any anatomical change in adipose tissue? 2. Are the biochemical changes a consequence of the expansion of the adipose tissue mass, or do they simply reflect adaptation to the changed intake of foodstuffs responsible for the obesity and its maintenance? 3. Is there any evidence in experimental obesity that normal subjects may adapt to high caloric intake by a change in metabolic efficiency? We will follow the lead of Rabinowitz in the *Annual Review of Medicine* of last year in referring to this study as the "Vermont Study."[2]

Three considerations are relevant to any discussion of obesity.

Definition of obesity.—Obesity is thought of as involving hypertrophy and hyperplasia of adipose tissue alone. However, in a study of young adult accident victims and matched controls, Naeye (12) found mean heart weight 140 percent greater than that of the controls, kidneys 25 percent, pancreas 45 percent, liver 45 percent, and spleen 10 percent greater. Only brains were essentially the same in weight. Findings were similar in 22 obese children. An increase in cellularity of approximately 20 percent together with an increase in cytoplasmic mass was found by line-sampling and planimetry in the enlarged organs. This, Naeye attributed to hyperinsulinemia. This suggests that obesity should be thought of as a more universal hyperplasia and hypertrophy than that of adipose tissue alone.

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[2] We acknowledge the collaboration of Dr. Phillip C. Kellherer and Dr. Charles M. Gluck of the University of Vermont, Dr. Joseph Glennon and Dr. George Bray (now at University of Southern California) at Tufts University, and Dr. Ralph Goldman of the U. S. Army Quartermaster Research and Engineering Center, Natick, Massachusetts.

Heterogeneity of obesity.—Certainly, anyone dealing with clinical aspects of obesity is impressed that there appear to be distinct types of obesity. The variety of syndromes of heritable obesity in animals is well brought out in the report of the second Brook Lodge Conference on Spontaneous Diabetes in Laboratory Animals (13). In man, sometimes a few members of an otherwise lean family are affected with an almost malignant form of the disorder. Vague (14) has called attention to android and gynecoid forms of obesity. Diabetes is associated with obesity in some families, and in others apparently not. Hirsch & Knittle (15) have pointed out that in some patients with obesity cellular hyperplasia of adipose tissue predominates, and in others increase in size of adipocytes. In Naeye's studies (12) referred to above, only approximately half the obese accident victims had the visceral hyperplasia and hypertrophy, and he also emphasizes that the disorder is heterogeneous.

What is normal?—We think of the concentration of a hormone as being normal provided it falls within a range commonly found in unstressed normal control subjects. Actually, we should speak of a value as being normal if it is appropriate to the particular stimulus or situation, and comparison must be made with findings in normals subjected to the same stimulus. Thus, to take an example from another field, a value for aldosterone excretion might fall within normal range but be entirely inappropriate and hence abnormal in a patient with hypervolemia and hypertension. The same criteria for normality have to be applied to the evaluation of the hyperinsulinemia often seen in obesity.

FINDINGS DURING DEVELOPMENT OF OBESITY IN MAN

Adipose tissue and muscle.—Hirsch & Knittle (15) have recently reviewed the natural history of adipocytes and emphasized that the number is defined early in life and that once defined it has proven difficult to affect cell number. It has been reassuring to find that in the Vermont Study increase in adipose tissue mass by as much as 100 percent by overeating produced a measureable change in cell size only, from 0.42 μg of lipid/cell to 0.78, and that cell number remained constant at about 32×10^9 cells. Grossly, the distribution of the fat gained in experimental obesity appeared to be mainly central (8), but in more recent studies (11) we have found that adipocytes of the gluteal region, the anterior abdominal wall, and the triceps area shared equally in the increase in cell lipid content. Again, there was no suggestion of a change in cell number, and the distribution of stored lipid apparently depends upon the original distribution and number of adipocytes. There was considerable variation within groups of individuals and significant differences from site to site in adipose cell size. The possibility cannot be excluded that the cycle of weight gain causes proliferation of young adipocytes too small and devoid of lipid for measuring by the Hirsch-Gallian technique, as emphasized by Hollenberg (16). It is also possible that more prolonged or lifelong obesity could increase cell number, and Bray (17) reported such an instance in a patient who became obese in adult life as a result of an hypothalamic tumor. We badly need other studies of cell number in obesity of diverse etiology. Salans, Knittle & Hirsch (18) have found in very obese subjects that at peak weight the enlarged fat cell was relatively resistant to the action of insulin on incorporation of carbon-14 from glucose into CO_2. It was not clear from this early study how great a role was played by change in intake of food and by change in the baseline glucose uptake. This response of the fat cell to engorgement has been emphasized as a possible explanation for the hyperinsulinemia of obesity and for the apparent peripheral resistance to insulin. In a group of five subjects and controls in the Vermont Study, the resistance in vitro to insulin was measured at baseline and at peak weight at two levels of intake of carbohydrate (11). At peak weight with a normal intake of carbohydrate (235 g) all subjects showed a diminished insulin sensitivity. Increasing carbohydrate intake to an average of 400 g increased basal glucose oxidation by adipocytes, but did not reverse the relative insulin resistance of the enlarged fat cells. Comparison was made with normal controls receiving proportionately the same intake of carbohydrate and calories. Rabinowitz & Zierler (19) have shown in young moderately obese subjects that when insulin is infused into the brachial artery of the forearm, there is resistance to its action both on the muscle drained by the deep veins and on the superficial drainage bed containing predominantly adipose tissue. Employing the same technique of perfusion we have found insulin resistance of the deep and superficial beds of the forearm in four out of five of the same subjects with experimental obesity in whom the above in vitro studies of adipose tissue were carried out (10). In contrast to the increase in basal glucose uptake noted by Rabinowitz & Zierler, mean basal glucose uptake was reduced at peak weight, in spite of an increase in mean fasting arterial insulin from 6.9 ± 0.03 to 10.3 ± 0.8μU/ml. The flux of amino acids was measured in some of these experiments,[3] and it was found that the inhibition by insulin of the release of branch-chain amino acids was also blunted. The finding that the basal concentrations of amino acids in the blood draining the deep structures was

[3] The analyses were kindly done by Dr. Philip Felig.

unchanged suggested that the blunting was not due to dilution of the muscle with fat cells.

Plasma lipids.—The plasma lipids in the Vermont Study (Table 1) remained within the normal range, but cholesterol and triglycerides increased significantly, as in spontaneous obesity. In contrast to the finding in spontaneous obesity, the free fatty acids in the plasma were reduced, so that they cannot be implicated as contributing to any possible peripheral resistance to the action of insulin. Plasma lipoproteins measured by electrophoresis were unaltered. It will, however, require the completion of studies with controlled variation in diet now in progress to disassociate the effect of change in food intake from any possible effect of the increased adipose tissue mass itself.

Glucose tolerance.—Glucose intolerance is a frequent occurrence in spontaneous obesity, but tolerance may be entirely normal in the face of truly massive obesity. In the Vermont Study the initial intravenous glucose tolerance, measured after relatively short periods of equilibrium at peak weight, was significantly diminished. When, however, peak weight is maintained for longer periods and carbohydrate intake is lowered it appears, from preliminary studies, that tolerance is not consistently altered. Again it is necessary to make comparison at lean and peak weights after extended periods of constant diet and activity before the question of impairment of tolerance following development of experimental obesity can be resolved. A definite answer to the question whether carbohydrate tolerance is altered in an experimentally obese volunteer in comparison with that in the lean control state under identical conditions of diet and activity must await completion of our current studies.

Suppressible and nonsuppressible insulin-like activity.—Before discussing hyperinsulinemia in obesity it is important to establish that the insulin is biologically active. In spontaneous obesity this has been shown to be the case by Samaan (20). By assay with isolated fat cells prepared by the method of Rodbell we have shown in a group of subjects with experimental obesity and moderate increase in fasting serum insulin that the insulin-like activity which is suppressible by anti-insulin antibody is also increased. Previous reports in spontaneous obesity have indicated that the nonsuppressible fraction, NSILA, may be increased in obesity and further increased by starvation (21). Its biological activity is a matter of debate. In experimental obesity we have found NSILA is significantly reduced at peak weight in comparison with the activity at basal weight.

Plasma immunoreactive insulin and the question of antagonism to the action of insulin in obesity.—Perley & Kipnis (22) clearly demonstrated that even though there may be apparent hyperinsulinism in lean diabetic subjects, the response of insulin secretion is less than that of normals given an identical hyperglycemic stimulus. Thus, it became unnecessary to postulate a circulating or tissue antagonist to insulin in lean diabetic patients. They found in the spontaneously obese, however, that identical hyperglycemic stimuli produced a greater-than-normal response, and many other workers have also found hypersecretion of insulin in response to a variety of stimuli in obesity (23).

Bagdade, Bierman & Porte (24, 25) have suggested that there is a basal insulin concentration and a secretion of insulin from a readily releasible pool in the obese and that this bears a fixed relation to the degree of adiposity. They suggest that correction can be made for this variable. However, it now appears that basal insulin and its secretion are affected by physical activity and by variation in intake of carbohydrate.

Björntorp and his colleagues (26) have found that following rigorous physical training the fasting insulin concentrations and the response of insulin to oral glucose of obese subjects return to the usual normal range. This occurred even though, surprisingly, their adipose tissue mass, calculated from distribution of tritiated water and radiopotassium and presumably the adipocyte size, was slightly increased and their glucose tolerance was unaltered. However, before one can say that the hyperinsulinism of obesity is completely correctable by physical training, comparison has to be made with the response of lean subjects similarly trained. That the reduction in basal insulin should occur is not surprising, since insulin is only one of the factors influencing transport of metabolic fuels into cells. Short et al (27) have described the anatomical and functional changes which take place in muscle following long-term exercise of a single limb in man. The elusive muscle activity factors described by Goldstein (28) and later by Havivi & Wertheimer (29) and perhaps even the NSILA of plasma (21) could also play a role.

A number of studies of insulin response to variations in intake of carbohydrate have shown the dissociation between body fat and insulin response and have shed light on the question of insulin antagonism in obesity.

Grey & Kipnis (personal communication) have recently given isocaloric diets containing either 70 percent carbohydrate or less than 15 percent serially to seven patients who were 70 percent or more overweight. Basal insulin ranged from 25 to 60 μU/ml initially, declined to 10 to 30 μU/ml during a three-week period of the low-carbohydrate diet, and promptly rose to initial concentrations on resumption of a high carbohydrate diet. Weight remained constant. Strictly comparable control data are not available. However, Danforth (personal communication) has fed isocaloric diets devoid of carbohydrate to five normal-weight volunteers. Plasma insulin became greatly reduced or almost undetectable, and the response of insulin secretion to glucose and arginine was decreased. Grey, Goldring & Kipnis (30) have also demonstrated lack of insulin responsiveness in starved rats refed diets devoid of carbohydrate. Grey & Kipnis (personal communication) had fed hypocaloric diets containing over 90 or 0 percent carbohydrate serially to

TABLE 2. The Effect of Starvation upon Blood Constituents and Nitrogen Excretion in Lean and Obese Subjects[a]

	Day 0		Days 8–10	
	Normal	Obese	Normal	Obese
Blood glucose mg/100 ml	77.0	89.2	63	68.2
Immunoreactive insulin μU/ml	14	45	8.3	20
Insulin/glucose ratio	0.18	0.51	0.13	0.29
Free fatty acids	0.53	0.71	1.88	1.36
Glycerol	62	90	164	110
β-Hydroxy butyric acid	0.016	0.07	4.23	4.30
Urinary nitrogen g/sq M/day	6.4		5.5	

[a] Data taken from Cahill et al (31) for lean subjects and from Felig et al (32) and Owen et al (33) for obese subjects.

three other obese patients. Mean basal insulin was 25, 23, and 45 μU/ml, was unchanged during two periods of high carbohydrate intake, and fell only to 12, 12, and 10 μU/ml, respectively, during the period of weight loss and of carbohydrate-free diet. Finally, the classic studies carried out at the Joslin Research Laboratory by Cahill and his group (31–33) enable one to compare the response of insulin to fasting in lean and obese subjects. The relative elevation of plasma insulin persists in the obese throughout eight to ten days of fasting, and it cannot readily be attributed to differences in circulating fuels known to stimulate insulin secretion. The relevant findings are given in Table 2. Plasma insulin falls in both and by a greater percent in the obese, but the final concentration remains elevated compared to that in the lean subjects. The insulin-to-glucose ratios behave similarly. Plasma glucose after fasting is somewhat higher in the obese. That the insulin is effective in the obese is suggested by the lower serum free fatty acids and glycerol. The degree of ketosis is comparable. Comparative data regarding plasma amino acid responses are not available.

Two interpretations can be made of the above data: 1. There must be some absolute antagonistic factor, since, in comparison with the normal under all the dietary conditions studied, insulin concentrations remain higher in the obese. 2. On the other hand, these patients with lifelong obesity have beta-cell hyperplasia and hypertrophy and therefore continue to respond excessively to stimulation after three to four weeks of an experimental diet. In an attempt to dissociate the effect of increased adipose tissue mass and increased intake of carbohydrate on insulin response, we are currently studying the response of volunteers to isocaloric diets containing two levels of carbohydrate at baseline and at peak weight. Since the obesity is experimental, each subject may serve as his own control.

The hyperinsulinism of experimental obesity appears to be of a lesser order of magnitude than that in many cases of spontaneous obesity, although we have as yet no sound basis for comparison. We have need of comparative studies of experimental and spontaneous obese subjects who are at least matched for degree of obesity, composition of diet, caloric balance, and activity, if not for duration. Since obesity is a heterogeneous disorder, as emphasized in the introduction, studies (particularly of the vexing question of antagonism to the action of insulin) will need to be on well-characterized patients. It would be helpful if we could separate out patients with a genetic predisposition to diabetes, but lacking a knowledge of the cause of diabetes or any biochemical marker, this cannot yet be readily accomplished. On the basis of what we have learned from the study of experimental obesity, we can conclude that development of such obesity probably would accentuate latent diabetes, but we cannot leap to the conclusion that true exogenous obesity alone could produce a diabetic state.

Progress in animal research on obesity would be difficult if work were carried out on a mixed bag of spiny mice, Egyptian sand rats, ob/ob Bar Harbor obese-hyperglycemic mice, Wellesley mice, Zucker fatties, and striped ground squirrels. Perhaps each has its human counterpart and if so more attention must go to defining the populations being studied.

In conclusion, we believe that the bulk of evidence favors some resistance to the action of insulin in obesity, but that the degree of resistance may be modified by variations in physical activity and in intake of carbohydrate.

Glucagon.—The physiologic role of glucagon in controlling glycogenolysis and gluconeogenesis seems well established. Its inappropriately high concentration in diabetes and the diminished suppression by glucose (34) is of great interest. In spontaneous obesity in children Paulsen & Lawrence (35) have reported high fasting concentrations of immunoreactive glucagon. This occurred mainly in those with a negative family history for diabetes but with abnormal glucose tolerance. Most of the elevated initial concentrations in the obese children were briefly suppressed by glucose, but surprisingly the control subjects did not show the expected fall in plasma glucagon following administration of glucose. The antisera used at the time of this study were not as specific as those now available, but reanalysis of the same sera with antiserum specific for pancreatic glucagon has confirmed the elevation in these patients (A. M. Lawrence, personal communication). The possibility that increased glucagon secretion might explain the biochemical changes in spontaneous obesity in animals has been most extensively explored in the Bar Harbor ob/ob mouse. Gepts, Christophe & Mayer (36) have found that the percent of pancreatic alpha cells is reduced in the young obese mice (8 percent as opposed to 17 percent in their lean littermates). However, since the total islet volume is increased approximately sixfold, there is an absolute increase in alpha cells estimated as threefold. Without knowing the actual glucagon secretion, it is not yet possible to say whether the absolute increase in alpha cells or the decrease in their proportion is the more important factor, or whether the changes are primary or secondary. More work is indicated in this promising area to determine whether glucagon may play a significant role in obesity.

Growth hormone.—The diminished responsiveness of secretion of growth hormone to various stimuli both in spontaneous and in experimental obesity is by now well documented (Table 1). The rise in serum growth hormone which normally occurs 4 to 6 hours after oral glucose ingestion is absent or blunted in spontaneously obese individuals (37, 38) as is the growth hormone response to insulin-induced hypoglycemia (38–40), arginine infusion (41, 42), and muscular exercise (37). That these changes are reversible has been demonstrated by Lessof, Young & Greenwood (39) and Kalkhoff, et al (43), who found return of normal growth hormone responsiveness to insulin-induced hypoglycemia following weight reduction, and by Ball and co-workers (44), who have recently reported normal growth hormone responses following oral glucose six months after maintenance of weight reduction in obese subjects.

Further evidence that decreased growth hormone secretion may be a secondary adaptive process to weight gain is derived from our studies of experimental obesity in which weight gain is associated with decreased growth hormone secretion following oral glucose ingestion, arginine infusion, and onset of sleep.

The physiological significance of decreased growth hormone secretion in obesity is not known. Since growth hormone is concerned with both conservation of protein (anabolism) and utilization of fat (anticatabolism), there is little need for its presence in the over-fed state. The net effect of diminished growth hormone secretion in obesity would be decreased rates of protein synthesis and lipolysis. Bray (45) has recently shown that in spontaneous obesity, administration of growth hormone has a calorigenic effect, and thus its suppression may limit calorigenesis and promote a positive energy balance.

Cortisol.—While the excretion of total cortisol and metabolites is increased in spontaneous obesity, it is well established that plasma concentrations are normal or reduced (9). The secretory rate is increased, as is the metabolic clearance rate. In experimental obesity in the Vermont Study, fasting plasma cortisol was found to be reduced at peak weight. When cortisol concentrations were measured at frequent intervals during the course of 24 hours in a subsequent group of volunteers, it was seen that the early morning drop in serum cortisol was more marked at peak weight, perhaps accounting for the difference. At other times throughout the day the values were entirely comparable in the obese and basal states. Thus, one cannot attribute the apparent increase in peripheral insulin resistance, if such actually exists, to increased action of cortisol. On the other hand, the fact that more cortisol is cleared through the liver may contribute to increased gluconeogenesis. The excretion of total 17-hydroxycorticoids was increased in the subjects with experimental obesity on an absolute basis, but not significantly increased when the values were related to body weight or surface area. Similar findings have been reported in spontaneous obesity not associated with Cushing's syndrome.

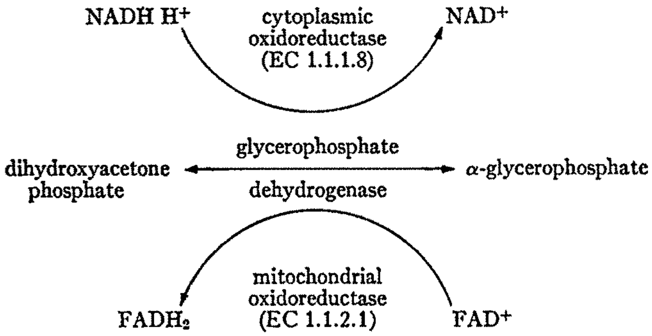
Functional derangements.—As pointed out above, the degree of physical activity has important effects on a number of biochemical parameter in man. Spontaneous physical activity is reduced in many and perhaps a majority of overweight persons (46), but whether this is a primary or secondary characteristic is not certain. Subjectively, the volunteers of the Vermont Study have had less drive when at peak weight and some have complained of slight dyspnea on exertion, but as yet decreased activity is not documented by objective measurements. At some time during the latter half of the day there is usually evidence of increased appetite associated with an aversion for breakfast, a familiar pattern in spontaneously obese patients. Reactive hypoglycemia has not been documented to explain this. It would be of great interest to know whether the changes in the patterns of appetite noted by Schachter in obese Columbia students (47) are also duplicated in the experimentally obese.

In the initial reports of the Vermont Study much emphasis was placed on the fact that most of the changes noted would tend to perpetuate the state of obesity. It was emphasized that these changes could represent reaction patterns which had survival value in more primitive times, when prolonged periods of fasting had to be endured. In our teleologic zeal we quite overlooked the fact that in spite of so many factors promoting lipogenesis, our subjects with few exceptions rapidly and spontaneously lost weight to their baseline values. This occurred with the same rapidity with which many obese patients who have managed to lose weight gravitate back to their original weight. As Liebelt (48) has pointed out, in animals there is apparently some overriding mechanism by which the body senses and controls its total adipose tissue mass and its state of repletion. Astwood asks in the address cited above, "So why can't heredity be credited with determining one's shape?" and one is inclined to answer in the affirmative.

Is Luxuskonsumption dead?—In the course of a 746-day balance study which he carried out on himself at the turn of the century, Neumann (49) noted that in spite of moderate variations in total caloric intake his weight remained constant without obvious change in activity. In 1912 Graefe and Koch (in 50) reported experiments involving overfeeding both of dogs and of undernourished clinic patients (not realizing that undernutrition lowers metabolic rate) which lead them to conclude that an increase in the metabolic rate in response to overfeeding is a normal mechanism to maintain constant body weight in spite of periodic excesses of food intake. Heimreich (in 50) reached a similar conclusion after studying the effect of doubling the caloric intake of children, as did Gulick in 1921 following extensive self-feeding experiments. This concept became known as Luxuskonsumption, literally, consumption of luxury, and until recent times has been regarded as a form of physiological heresy. Many prestigious investigators (50) including Wiley & Newburgh (6) showed that aside from the specific dynamic action of protein, overfeeding did not increase the basal metabolic rate, and this observation has not been contested. Passmore and his co-workers (5) carried out careful experiments on the effect of overfeeding three young men 1300–1600 excess kcal per day for 10 to 14 days, and after carefully estimating energy balance throughout the day could find no shred of evidence for Luxuskonsumption. Studies of overfeeding two obese women yield similar results. Recently, seven normals and nine overweight subjects were fed 3000–8000 kcal in total excess over a four-day period and meticulously studied; again there was no evidence to support Luxuskonsumption, but the studies were of short duration and involved modest caloric excess (5).

Early in the Vermont Study we were impressed with the fact that there was poor correlation in our volunteer weight-gainers between the caloric intake achieved and the actual gain in weight. As can be seen in the diagram of caloric intake for weight gain for subject R. F. of the initial reports (8, 9), a marked excess of calories was necessary to achieve the gain in weight. It was estimated only very approximately from standard dietary tables that in the last two groups of volunteers 1800 kcal/sq M was required to maintain weight during the basal period, but that at peak weight approximately 2700 kcal/sq M were required to maintain constant weight. This is in contrast to the 1100–1300 kcal/sq M usually required for spontaneously obese subjects when hospitalized. The difference of approximately 1400 calories per day could not be readily accounted for by change in activity, increased work of transporting the added weight, probable fecal loss, or systematic error. A number of recent observations in animals suggest that there may be variations in efficiency to explain discrepancies between caloric intake and increase in body stores of energy. In 1962, Miller & Payne (51) found that weight could be kept constant in rats given a wide range of caloric intake with constant protein intake, even though there was no change in activity or intestinal absorption. Over a three-week period, rats induced to take excessive calories expended as heat calories equivalent to their total carcass energy (690 kcal). Similarly, over a 40-day period, pigs maintained their weight on either a 243- or 1185-calorie diet without change in measured gross activity or fecal loss. The change in stored energy of the animals was determined by analysis of the carcasses in control littermates and the experimental groups. In 1967, Miller, Mumford & Stock (52) carried out short-term experiments with high caloric intake in human volunteers which lead them to the same conclusion. The subjects ate an excess of 1530 kcal per day for three weeks. There was no consistent change in BMR, but the consumption of oxygen was considerably raised while overeating. The increment was mainly during periods of exercise and also in response to eating. They point out that Passmore's estimates did not take into account the increase in oxygen consumption following meals.

There are two situations in which heat production is increased. First, there is the well-known relatively increased heat production of hyperthyroidism. Secondly, it has been known for some time that in adaptation to cold thermogenesis is increased, efficiency of utilization of food is decreased, and a larger proportion of the calories ingested is dissipated as heat. In their classic review of cold adaptation Smith & Hoijer (53) suggested in 1962 that while oxidation still remained tightly coupled to generation of high-energy phosphate bonds, certain substrates, notably succinate, certain acyl compounds, and α-glycerophosphate might be preferentially oxidized through pathways which yielded a P:O ratio of less than 3, so that "efficiency" is reduced and a greater proportion of calories is released as heat. An example of this is given by the so-called α-glycerophosphate cycle, which shuttles hydrogen from NADH in the cytoplasm to FAD in the mitochondria, allowing the hydrogen to enter the respiratory chain at a point beyond which only two high-energy phosphate bonds are generated.



In 1964, Chaffee, Smith & co-workers (54) demonstrated that one of the major biochemical changes associated with adaptation to cold in the hamster was an increase in respiration associated with α-glycerophosphate in the liver and to a much greater extent in brown fat. Thus, one or more mechanisms exists for a similar change in efficiency of utilization of foodstuffs which aids in the regulation of body energy stores.

In 1967, Stirling & Stock (55) repeated the original experiments of Miller & Payne described above in which rats were induced to take a diet with excess calories as fat and carbohydrate, and showed an increase in the mitochondrial FAD-dependent α-GPDH (1.1.2.1.) in liver. The response of oxygen consumption to injection of norepinephrine was also increased in the rats in which increased thermogenesis had been induced. Galton & Bray (56) have studied α-glycerophosphate metabolism in patients with obesity. They found the activity in adipose tissue of both the cytoplasmic and the mitochondrial α-GPDH to be diminished in severely obese subjects in comparison with that in lean control subjects, and concluded that this promoted lipogenesis by increasing the α-glycerophosphate available for conversion to glycerol and triglyceride. Several patients with obesity of hypothalamic origin did not show this difference. In a later paper, Bray (57) suggested that difference in the activity of the α-glycerophosphate cycle might explain difference in the efficiency with which obese patients utilize their food. He found that the activity of the two dehydrogenases in grossly obese patients was reduced by caloric restriction, but that this reduction of the mitochondrial enzyme was prevented by relatively large doses of triiodothyronine.

The evidence is still not adequate to say whether limitation of the activity of the mitochondrial enzyme might be an important primary biochemical lesion in obesity. Situations in which deviations from normal have been observed to date have been those involving acute change in diet. Whether failure of adaptive thermogenesis is important in the day-to-day balance of an obese patient remains to be determined. If such a mechanism is operative, it is presumably only one of several factors controlling the size of the total mass of adipose tissue. Further studies are clearly indicated, and it will also be important to establish with precision whether thermogenesis is increased in normals following overfeeding, and whether α-GPDH is increased by high caloric intake. Bray (58) has recently emphasized the futility of treating obesity by diet alone, pointing out that under conditions of caloric restriction the calories needed to maintain weight may drop from an initial 1100 or more kcal/sq M to less than 900 kcal/sq M. Since such restriction reduced mitochondrial α-GPDH activity, the efficiency of utilization of food for the generation of ATP remains high. Since under the carefully controlled conditions of a hospital ward he had found that tri-iodothyronine in large doses (250 μg/day) restored the activity of the enzyme to that of normals, he has cautiously and circumspectly suggested that "some therapeutic modalities that have come under recent criticism may need re-evaluation as potential agents in our search for more effective ways of dealing with obesity."

SUMMARY

We will again borrow a quotation from Dr. Astwood's 1962 address (1) which incorporates the feeling of our group:

"One can also fatten animals by stuffing and doubtless we could do the same thing to ourselves if we put our minds to it.... But I do not consider this a common cause of overweight in man; not many people try to get into the circus this way—they become candidates spontaneously."

In the eight years since this was written several things have become evident:

First, those who do become candidates for the circus, the severely obese, are seriously handicapped by an increased number of adipose tissue cells. In short-term experimental obesity, however, no increment in adipocyte number can be detected.

Secondly, people can fatten themselves by putting their minds to it, but when they do, they develop most of the biochemical changes seen in spontaneous obesity to a minor degree. But from this it should not be said that these changes, when they occur in spontaneous obesity, are necessarily the result of the increase in adipose tissue mass.

Thirdly, there may well be mechanisms, specifically those involving adaptive increase in nonshivering thermogenesis, which tend to minimize the gain in weight of a normal person as a result of increased intake of food. When normal people no longer put their minds to gaining excess weight they gravitate toward their habitual weight.

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SMALLPOX AND SMALLPOX VACCINATION POLICY

7016

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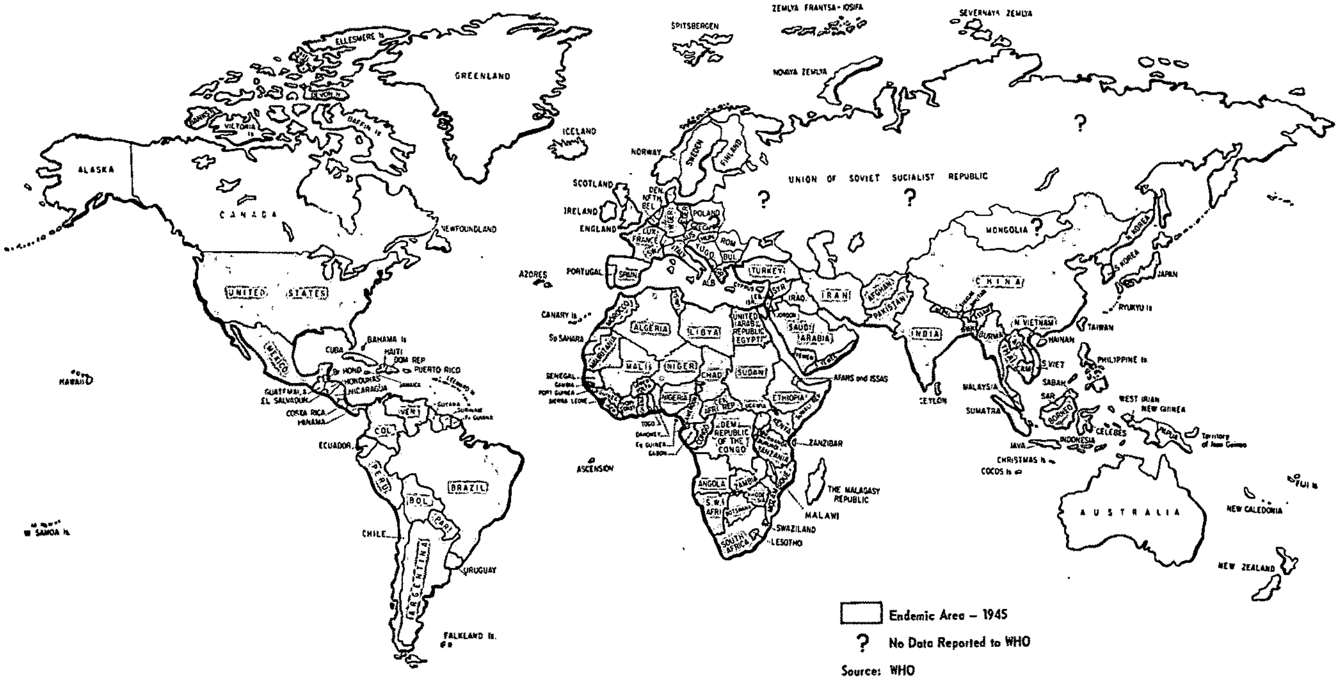
INTRODUCTION

Smallpox is being rapidly reduced throughout the world, primarily by smallpox eradication campaigns. The introduction of heat-stable lyophilized smallpox vaccine, coupled with the World Health Organization's drive against the disease, has made vaccination widespread and effective. In the past 25 years, smallpox has been eliminated from North America, Western Europe, Russia, China, North Africa, the Middle East, much of Southeast Asia, most of the new countries of sub-Saharan Africa, and all of South and Central America except parts of Brazil. Formal smallpox eradication campaigns have been initiated in all endemic countries. Figure 1 presents maps of the distribution of endemic smallpox in 1945 and in the first eight months of 1970 (1).

In the United States, defense against smallpox has been based upon four principles. First is routine vaccination of the population. Second is vaccination of travelers. Third is inspection of vaccination certificates of returning travelers and immigrants. Fourth is investigation of suspect smallpox cases, with rapid isolation and control of smallpox importations. Approximately 15 million smallpox vaccinations are performed in the United States each year, about six million of which are primary vaccinations. The total cost of the United States for smallpox prevention, including vaccine production, vaccination fees, time lost from work or school for vaccination, maintenance of the foreign quarantine system, research on smallpox and vaccinia, and medical costs of vaccination complications, is an estimated $147,000,000 (2).

There is disagreement about the need for and effectiveness of current United States policies. Kempe has emphasized the dangers of smallpox vaccination (3). Dick believes that, in Great Britain, routine population vaccination is less effective and more costly than surveillance of disease introductions and outbreak control (4). The United States Public Health Service's Advisory Committee on Immunization Practices, and the American Academy of Pediatrics Committee on the Control of Infectious Diseases recommend routine childhood vaccination. Most state and local health de-

Figure 1
WORLD-WIDE SMALLPOX
ENDEMIC AREAS —1945

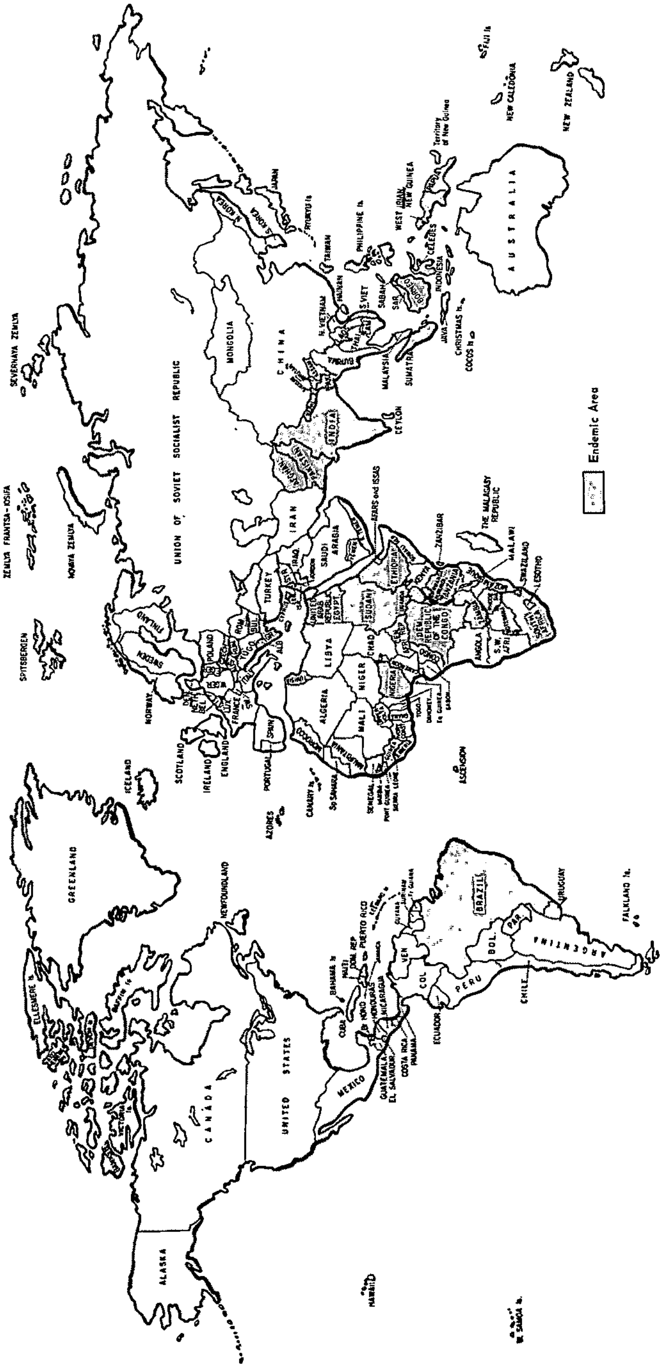


☐ Endemic Area – 1945

? No Data Reported to WHO

Source: WHO

Figure 1
COUNTRIES REPORTING ENDEMIC SMALLPOX
JANUARY—AUGUST 1970



partments include routine vaccination as part of well child care, and many school districts demand vaccination for school entry.

There are at least four factors to consider when forming a rational vaccination policy. First is the immunology of smallpox and vaccinia. What are the basic mechanisms of immunity and how long does it last? Second is the risk of vaccination. What are the types of vaccination complications? How frequently do they occur? What are their probable rates of morbidity and mortality? Can these risks be reduced? Third is the epidemiology of smallpox. How does it spread, who gets it, how rapidly does it spread? Fourth is the risk of importation and spread of smallpox in the United States. How often can we expect an importation, and how many cases and deaths might result from an importation?

In this chapter we shall attempt to quantitate and weigh these factors and try to answer the question, Should routine childhood vaccination be continued? The reader should understand that the answers to this question may change from time to time and from place to place. Our attempts will be based on the position of the United States in 1970.

IMMUNITY PRODUCED BY VACCINATION

General Considerations

Immunity against variola and vaccinia depends upon cellular immunity, circulating antibodies, and perhaps local factors such as the production of interferon. The pathophysiology of variola is believed to be similar to ectromelia or mousepox (5). There is viral multiplication at the site of invasion, a primary viremia, viral multiplication in the reticuloendothelial system, a secondary viremia, and, finally, the virus localizes in the skin and certain other organs, where multiplication results in the characteristic rash (6) and associated pathology. The initial viremia of smallpox may be aborted by neutralizing antibodies. Cellular immunity mechanisms may inhibit or prevent replication of the virus at the site of invasion.

Vaccinia infection differs from smallpox infection in that virus replicates at the site of inoculation, and perhaps extends to regional lymph nodes, but viremia does not regularly occur after uncomplicated primary vaccination (7). Vaccinia virus may therefore be less accessible to the action of circulating antibody than is variola. Several lines of evidence indicate that in vaccinial infections the principal line of defense is the cellular immunity system. First, Vaccinia Immune Globulin (VIG)[1], which contains primarily 7S gamma globulin, can modify and in some instances prevent smallpox, but does not affect the local response to vaccination (8). Second, individuals with the Bruton-type hypogammaglobulinemia, who have intact cellular immunity mechanisms, can respond normally to vaccination despite absence of circulating antibodies (9). Third, progressive vaccinia (vaccinia necrosum) occurs primarily in patients with impaired cellular immunity; it may occur when circulating antibodies are present, and may be refractory to treatment with VIG (10–12). Fourth, cellular immunity mechanisms can be dissociated from circulating immunoglobulin in laboratory animals, and it has been shown that the cellular immunity system is most important for recovery from vaccinia (13).

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[1] Inclusion of trade names is for identification only, and does not imply endorsement by the Public Health Service of the U. S. Department of Health, Education, and Welfare.

The role of local factors, particularly interferon and secretory IgA, in recovery from vaccinia or smallpox has not been defined. Vaccinia is a good inducer of interferon, and is susceptible to the action of interferon in vitro (14). Smallpox inoculated into the skin (variolation) apparently does not produce as long-lasting immunity as does the natural disease (15).

IMMUNITY CONFERRED BY VACCINATION

The response to classical intradermal primary vaccination is well established. Circulating antibodies (HAI and neutralizing antibodies) can be detected within five days after vaccination, and increase in titer for two to four weeks thereafter (16). Such antibodies, particularly neutralizing antibody, may persist for several years. Delayed hypersensitivity, as indicated by modification of the cutaneous reaction to serial vaccinations, can be demonstrated as early as four days after primary vaccination. The duration of cellular immunity has not been established, but the dermal response to revaccination may show modification as long as 20 years after primary immunization.

The overwhelming evidence that vaccinia protects man from variola need not be reviewed here. Successful vaccination reduces the risk of acquiring smallpox to virtually nil for from three (Variola major) to seven (Variola minor) years (17, 18). The contrast between the probability of vaccinated and unvaccinated individuals acquiring smallpox decreases with time so that 20 years after vaccination there is little protection left from vaccination (19). Primary vaccination in the incubation period affords some protection if given within the first three or four days after exposure (20). Revaccination of a partially immune individual in the incubation period may be effective in aborting the disease when performed as late as seven or eight days after exposure (21). There is considerable individual variation in the levels of circulating antibodies and the correlation of the level of circulating antibodies with attenuation of the dermal reaction to vaccinia has not been established.

CURRENT UNITED STATES VACCINATION POLICY

The effectiveness of the current United States vaccination policy in preventing the re-establishment of smallpox is difficult to quantitate. There has been no challenge of the population's immunity since 1949. The vigilance of the foreign quarantine program, the enforcement of vaccination requirements for foreign travel, and the paucity of travelers to the United States from smallpox endemic countries and vice versa have maintained our freedom from importation. Our population's immunity cannot be credited with preventing importations.

Several surveys demonstrate that the immunity level in the United States population is relatively low. Seven percent of the United States population is vaccinated each year. Eleven percent of the population has never been vaccinated. Over half of the vaccinations performed each year are revaccinations, and considerably less than 20 percent of the population has been vaccinated within three years (22). Surveys of special population groups, especially hospital workers, indicate that fully 50 percent of these populations have not been vaccinated for 20 years (23-25). Therefore, 11 percent of the population has no immunity to smallpox, and perhaps 50 percent of the population has insufficient immunity to prevent the disease. Considering the difficulty of quantitating individual immunity, and the lack of data on the vaccination history of the population, precise estimates of the population immunity cannot be made. We believe that elimination of the sources of smallpox in other countries will do more to protect the United States than will maintenance of this uncertain immunity in the United States population.

Individuals belonging to certain groups are at particularly high risk for contracting smallpox, either if it is reintroduced into the United States or if it is not eradicated throughout the world. These groups include health workers, military personnel and travelers to endemic countries. Studies of the epidemiology of introduced smallpox indicate that health workers are at high risk for acquiring disease (26). Military recruits must be vaccinated because of the unpredictability of the geography and environment of their assignments. The rationale for vaccination of travelers to endemic areas is obvious. We have estimated elsewhere that if the entire population were unimmunized, approximately 1.4 million adult primary vaccinations would have to be performed annually among these three groups (27).

HAZARDS OF VACCINATION

General Comments

Throughout the latter part of the 19th and the early 20th Centuries, occasional complications to vaccination were noted and accepted as an insignificant risk compared to the risk of smallpox. Reporting of complications was poor. After the 1930's, as smallpox became rare in many Western nations, more attention was paid to the hazards of immunization. In 1946, a report was made concerning postvaccinial encephalitis to the Interim Commission of the World Health Organization (28). Later, complications of vaccination became reportable diseases in England, Wales, and other European countries.

Vaccination complications are not routinely reported in the United

States, and, except for Greenberg's report of complications in the 1947 New York City smallpox outbreak (29), quantitative data from this country were not available until recently. In the late 1950's and early 1960's, Kempe repeatedly pointed out the hazards of vaccination (3, 8). In the last few years, we have studied the frequency and characteristics of vaccination complications in the United States (11, 30–33).

Historically many vaccination complications arose from the use of contaminated vaccines, the use of lymph from human Jennerian vesicles, or the use of poultices or occlusive dressings applied to the vaccination site. These practices caused many deaths from streptococcal or staphylococcal sepsis, tetanus, and other bacteria (34). Occasionally, when lymph from luetic or lepromatous patients was used to vaccinate others, outbreaks of syphilis and leprosy even occurred (34). Modern vaccines and vaccination techniques have virtually eliminated these occurrences, although references to them are still found in the more lurid antivaccination literature. It is not our object to review bacterial complications, but one should note that one case of tetanus occurred in the United States in 1967 in whom the portal of entry was apparently a smallpox vaccination (35).

DEFINITIONS AND CLINICAL CONSIDERATION

Complications of smallpox vaccination can be divided into three categories: central nervous system, dermal, and "other." The most common central nervous system complication is postvaccinial encephalitis. The dermal complications include accidental infection, erythematous urticarial eruptions, generalized vaccinia, eczema vaccinatum, and vaccinia necrosum. The "others" include fetal vaccinia, a variety of rare inflammatory conditions such as myocarditis, arthritis, etc., and perhaps sudden death in infancy.

Postvaccinial encephalitis.—Postvaccinial encephalitis has been defined as a central nervous system illness following vaccination. It usually occurs in primary vaccinees. The frequency of occurrence and age distribution vary greatly from country to country (28).

A wide variety of symptoms and signs have been described with postvaccinial encephalitis including meningismus, convulsions, coma, muscular paralysis, polyneuritis, brachial neuritis, myesthenia, transverse myelitis, and simply drowsiness and lethargy (30, 32, 36, 37). Most patients with severe postvaccinial encephalitis present with fever, and signs and symptoms caused by increased intracranial pressure. The spinal fluid in some patients is normal, while in others varying pleocytosis and/or protein elevation is present. Most cases occur 7 to 14 days after vaccination, but cases have been reported as early as one day and as late as 23 days following vaccination. When death occurs, pathological findings frequently encountered are cerebral edema and perivascular mononuclear infiltration of cerebral vessels with some degree of surrounding demyelinization. These findings may not be present in very young infants (36). Since attempts to isolate vaccinia from CNS tissue are generally unsuccessful, the pathogenesis of postvaccinial encephalitis is felt to be similar to other postinfectious encephalitides, such as that following measles. A true infection of central nervous system tissue with vaccinia virus may occur in rare patients.

The problem of postvaccinial encephalitis cannot be easily reduced because it occurs in normal individuals who cannot be identified and denied vaccination by any currently known screening techniques. Studies of Dutch Army Recruits indicate that the incidence of postvaccinial encephalitis (PVE) can apparently be decreased by the administration of VIG before vaccination (38), and that vaccination with an attenuated strain of vaccinia virus in conjunction with administration of VIG may further reduce the incidence (39).

Treatment is supportive and should be directed toward reducing cerebral edema. Recovery is usually complete, although residual neurological or psychiatric defects have been documented (32).

Accidental infection.—Accidental infection is the accidental or aberrant implantation of vaccinia virus in individuals who do not have a pre-existing skin disorder. The inoculations may occur from contact with a recently vaccinated person, from autoinoculation of vaccine transferred from the site of vaccination, or of virus acquired from scratching the primary lesion. The most common sites of inoculation are the face, the palpebral margins of the eyelids, nose, or mouth (30, 32). Inoculation of the genitalia or anus is also common. These lesions are generally self-limited, but severe infections may be treated with vaccinia immune globulin. Corneal inoculation may occur if excoriation of the cornea is present before the implantation of vaccinia (40).

Patients with corneal involvement may benefit from idoxuridine ophthalmic drops or ointment. Although VIG is often used, most patients recover spontaneously with no therapy.

Generalized vaccinia.—Generalized vaccinia is a common but poorly defined complication of vaccination, characterized by a vesicular rash of varying extent. It probably does not always result from general dissemination of vaccinia virus as the name implies, for virus is rarely isolated from the peripheral lesions. In patients without a pre-existing dermatologic disorder or antibody deficiency syndrome, the illness is quite mild and causes little or no residual scarring. The onset of the rash generally occurs 7 to 12 days after vaccination (30, 32). Generalized vaccinia occurs primarily in primary vaccinees and is more frequent in infants than in older children (30, 32). The pathogenesis is unclear; it may be a hypersensitivity syndrome, since a viremia is apparently rare. Antihistamines may offer some symptomatic relief.

When systemic signs and symptoms lead one to suspect blood-borne virus dissemination, VIG may be of benefit. The frequency of generalized vaccinia can be decreased by delaying vaccination until after the first birthday, but no definitive preventive measure is known.

Erythematous urticarial eruptions.—A generalized urticarial eruption, similar to erythema multiforme, frequently follows primary vaccination of normal individuals. The rash is mild, self-limiting, and rarely accompanied by fever. It occurs more frequently in infants than in older children (30, 32). The rashes are generalized and can be maculopapular or morbilliform. They may be similar to "generalized vaccinia" except that the lesions do not vesiculate. The onset generally occurs 7 to 12 days after vaccination. Occasionally patients develop a severe bullous exudative erythematous eruption similar to Stevens-Johnson syndrome.

A wide variety of confusing synonyms have been applied to these rashes, including roseola vaccinia, toxic erythema, postvaccinial urticaria, erythema multiforme, etc. We believe that there is insufficient evidence of differing pathogenesis or prognosis to justify such complex nomenclature.

There is no known preventive measure for these rashes. Therapy includes antihistamines, but care should be taken not to overtreat. Patients with bullous eruptions may be quite ill and require careful fluid and electrolyte balance, steroids, and supportive management.

Eczema vaccinatum.—Eczema vaccinatum is the local or systemic dissemination of vaccinia virus in individuals with eczema or a past history of eczema. This complication can occur after vaccination or after intimate contact with a recently vaccinated individual. Although most common and most severe in nonimmune persons, it occasionally occurs in revaccinees (30, 32). Many patients are quite ill and may die. Necropsy examination of such patients generally reveals vaccinia throughout most organs (41). The illness may be mild with only a few vesicular lesions and recovery rapid without therapy. Eczema vaccinatum is most severe when it follows contact infection rather than direct vaccination (32). Patients with severe eczema are seldom intentionally vaccinated, so that the unvaccinated contact group is biased by inclusion of the more severe eczema patients. In contact vaccinia, the virus may be simultaneously inoculated in several sites over many areas, causing severe and extensive disease.

Eczema vaccinatum can be prevented by withholding vaccination from individuals with eczema, a past history of eczema, or household contacts with eczema. When vaccination is indicated because of possible contact with smallpox, VIG should be administered prophylactically at the time of vaccination. We know of no failures of this form of prophylaxis.

The recommended treatment of severe eczema vaccinatum is VIG in a dose of 0.6 ml per kilogram, repeated if necessary. If this is not rapidly effective, particularly if new lesions continue to occur, N-methylisatin βthiosemicarbazone (Marboran)[1] may be beneficial (32, 42). Severe eczema vaccinatum is a potentially fatal illness and Marboran is an experimental drug; consultation should be sought for severe cases.

Vaccinia necrosum.—Vaccinia necrosum (progressive vaccinia) is a rare, often fatal complication of vaccination, characterized by progressive necrosis in the area of vaccination, often with metastatic lesions. Although the lesions can be extensive, patients may not have fever, erythema, pain, or regional lymphadenopathy, and may not come to their physician's attention until several weeks after vaccination.

Patients who develop vaccinia necrosum are deficient in cellular immunity mechanisms (8, 10, 11). They generally have deficient or defective white cells and/or immunoglobulins, or are receiving corticosteroids, radiation, or other immunosuppressive therapy. Patients with the Swiss type of agammaglobulinemia, in which cellular immunity as well as immunoglobulins are deficient, nearly always die when they develop vaccinia necrosum.

Vaccinia necrosum can be prevented by careful screening of patients for immunologic deficiency diseases. Therapeutic measures include administration of VIG in frequent and large doses, Marboran, and on occasion transfusions of whole blood or white blood cells from recently vaccinated donors. Topical idoxuridine ointment has also been used. Clinicians who are not experienced with this disease should seek consultation in planning therapy.

Other dermal complications.—Other dermal complications include secondary bacterial infection of the site of vaccination, and vaccinia infection of exudative skin diseases such as recent burns, diaper rash, impetigo, poison ivy, acne, etc. Vaccinia immune globulin may be indicated for treatment of severe or extensive involvement.

Other uncommon complications.—Conybeare (43), Apostolov and associates (44), and de Vries (45), have described a syndrome suggestive of overwhelming viremia following vaccination, and resulting in sudden death in infancy. The age at vaccination of their four patients was between two and five months. Onset occurred suddenly on the eighth or ninth day following vaccination, with hyperpyrexia as the major symptom. Death followed within 24 hours of onset. Vaccinia virus was isolated from multiple organs of one patient.

Since 1932, there have been 49 reports of fetal vaccinia. Nearly all the mothers received a primary vaccination or had a primary type of reaction accompanied by symptoms of systemic illness. The time of maternal vaccination ranged from the 3rd to the 24th week of gestation, and delivery usually occurred 3 to 13 days following vaccination. Nearly all of the fetuses were stillborn, or died shortly after delivery (46). They had multiple vaccinial lesions from which the virus could be isolated when isolation was attempted. The rarity of this complication may be related to the combined infrequency of primary vaccination in pregnant women and the infrequency of viremia following vaccination. When exposure to smallpox is unlikely, vaccination should be avoided in gravid nonimmune females. When vaccination is necessary, VIG may be added to reduce the likelihood of viremia and transplacental passage.

Several studies, most of which are poorly designed, have presented data on the incidence of congenital anomalies, stillbirths, and prematurity in infants born to vaccinated mothers (46–48). These studies do not convincingly demonstrate an increased risk of fetal wastage or deformity following maternal vaccination.

Individual case reports have linked vaccination to a variety of other illnesses, including myocarditis, thrombocytopenia, arthritis, and pericarditis (49–53). These may be etiologically related to smallpox vaccination, but their rare occurrence makes it impossible to rule out a mere chance relationship. Several cases of malignant melanoma arising in vaccination scars have been reported (54). There are several reports that vaccination of patients with lepromatous leprosy is often followed by acute erythema nodosum leprosum or lepra reactions (55–57).

INCIDENCE RATES OF COMMON VACCINATION COMPLICATIONS

Estimates of the frequency with which the different vaccination complications occur are based upon data of varying quality. Rates vary from one country to another and probably reflect differences in reporting as well as factors such as vaccine strains and the vaccination history of the population. The United States rates of postvaccinial encephalitis are considerably lower than those of some Scandinavian countries (28), but are generally consistent with those reported from England and Wales (30, 32, 43).

The frequency and mortality of severe complications in the United States have been estimated by following the distribution of VIG (30, 32). More precise figures on the less severe complications are derived from physician surveys or closely studied clinic populations (31, 33, 58). Table 1 presents the incidence rates for the various complications of primary vaccination based on modern United States studies. The rates for encephalitis, vaccinia necrosum, and deaths are taken from national surveillance data. The rates for eczema vaccinatum, generalized vaccinia, and accidental infection come from direct physician surveys. The rates for erythema multiforme (erythematous urticarial reactions) are from direct observation of children at a large comprehensive child care center.

Several observations are consistent throughout most studies of the incidence of smallpox vaccination complications. Complications occur at least ten times more frequently in primary vaccinees than in revaccinees. Complications in revaccinees occur either in individuals who have not been vaccinated for many years and therefore react like primary vaccinees, or in indi-

TABLE 1. COMPLICATION RATES OF PRIMARY SMALLPOX VACCINATION IN THE UNITED STATES, BY AGE AND DIAGNOSIS

Complication	Complications per Million Primary Vaccinations			
	Age at Vaccination			
	<1	1–4	5–19	20+
Death[a] (from all complications)	5	0.5	0.5	unk
Post-Vaccinial Encephalitis[a]	6	2	2.5	4
Vaccinia Necrosum[a]	1	0.5	1	7
Eczema Vaccinatum[b]	14	44	35	30
Generalized Vaccinia[b]	394	233	140	212
Accidental Infection[b]	507	577	371	606
Erythematous Urticarial Reactions[c]	unk	9600	unk	unk

[a] See references 30, 32. [b] See reference 33. [c] See reference 58.

viduals who have acquired diseases affecting their immune mechanisms. Deaths occur once or twice per million primary vaccinations (approximately eight deaths annually in the United States). The mortality and morbidity resulting from vaccination are higher in infants than in older children. Many patients are hospitalized, particularly with the more serious complications, resulting in at least 2000 annual patient hospital days in the United States.

Some studies show increasing risk of primary vaccination with increasing age (28). Recent data from England indicate that the risk is greater during infancy than during adulthood (43). There are too few adult primary vaccinations performed in the United States to enable quantification of the risk. Data from other countries indicate that the risk of death from vaccination in adults is at least twice that found in children. Recent studies in Great Britain indicate that the deaths per million adult primaries may approach three per million (43).

PREVENTION OF VACCINATION COMPLICATIONS

Many vaccination complications are preventable if the well-known contraindications to smallpox vaccination are carefully observed. The following individuals should not be vaccinated: those with an exudative skin rash, eczema, or a past history of eczema; those with a household member who has eczema or an exudative skin disorder; pregnant women; those with an antibody deficiency disease; those with leukemia, lymphoma, Hodgkin's disease or other blood dyscrasias; those being treated with immunosuppressive agents, corticosteroids, or radiation therapy. Infants should not be vaccinated if they have no immediate risk of contracting smallpox, because of their increased risk of complications. Care should be taken to wipe excess vaccine from the arm after vaccination to diminish the risk of contact vaccination or accidental infection. If these recommendations are closely followed, the frequency of severe complications to vaccination might be reduced by nearly half (30, 32). These contraindications have been known for many years, however, and a certain amount of human error may be unavoidable. There was no decrease in the complication rates in 1968 as compared to 1963, despite the wide publicity given Kempe's work and the National Communicable Disease Center's studies on vaccination complications.

ALTERNATIVES TO VACCINATION

Three alternatives to vaccination as the primary method to control smallpox are currently in existence. These are the use of vaccinia immune globulin (VIG), the use of Marboran, and the use of "attenuated" strains of vaccinia virus. These three agents have some promise, but their current level of development precludes their use as substitutes for vaccination.

VIG will reduce the attack rate of smallpox in treated versus untreated contacts of smallpox patients (59). Some contacts given VIG still acquire smallpox. VIG is expensive and in short supply because it is made from the blood of recently vaccinated human donors. We believe that VIG should be given, in conjunction with vaccination, to selected smallpox contacts with contraindications to vaccination, and also to individuals who are not vaccinated until several days after exposure to smallpox.

Marboran can reduce the attack rate of smallpox in treated versus untreated contacts of smallpox patients (60, 61). The drug's action is of short duration, and therefore it can only be used when patients are known to have contact with smallpox. Some patients treated with the drug still acquire smallpox. The toxicity of Marboran is largely unknown. Initial preparations of the drug caused marked gastrointestinal irritation. We believe that Marboran is useful for treatment of selected vaccination complications. It may also be useful for smallpox contacts with immunologic defects, such as leukemic patients on a ward with a smallpox patient.

Several workers have expressed hope that attenuated vaccinia strains may greatly reduce the rate of vaccination complications, and thus reduce the need to curtail routine vaccination. Candidate strains, particularly the CVI-78 strain, have some indications of reduced virulence for man (39, 62, 63). At present two problems preclude substitution of the CVI-78 strain for those currently used. First, the very low rate of vaccination complications makes a comparison trial between attenuated strains and the current strains essentially impossible. A comparison trial would have to include several million vaccinees to achieve statistically significant results since the death rate from vaccination complications is only per million primary vaccinations. There is no reason to believe that the incidence of postvaccinial encephalitis depends upon the virus strain rather than upon the allergic or immunologic idiosyncrasies of the vaccinee. Second, there is so little smallpox remaining in the world that a field trial of the protective effect of a new strain is essentially impossible. The use of "attenuated" strains is thus limited to primary vaccination, with certification of immunity only after revaccination with a fully virulent strain. Such a cumbersome procedure is unlikely to gain wide use. We believe work should continue on attenuated strains, but they hold little promise for solving the problems of smallpox control in smallpox-free nations.

EPIDEMIOLOGY OF SMALLPOX

Smallpox is uniquely susceptible to total world-wide eradication. There is no host or reservoir of the disease other than man. There are no subclinical carriers. Only close, generally identifiable, contacts of cases acquire the disease. Patients with sufficient infection to transmit the disease are overtly ill; even those with attenuation of the disease from prior vaccination get marked prodromal symptoms although their rash may be minimal.

The pathophysiology of smallpox is believed to be similar to that of ectromelia, or mousepox (5). The virus is acquired by inhalation of tiny droplets of aerosolized virus from sick patients. Virus is inhaled and infects the bronchiolar and upper respiratory tract epithelium. The virus multiplies in epithelial cells and macrophages, and then localizes in regional lymph nodes, tonsils, and adenoids. A primary viremia then occurs which ends when the cells of the reticuloendothelial system clear the virus from the bloodstream. In the R-E cells, virus once again multiplies extensively and is released in a secondary viremia, this time in massive amounts. The secondary viremia causes fever, myalgia, and malaise, and results in infection of skin and internal organs, giving rise to the characteristic exanthem and enanthem. In man, approximately 12 days elapse between initial infection and the secondary viremia, and approximately 14 days from initial infection to onset of clinically apparent rash.

There are several variants of smallpox virus which are antigenically indistinguishable, but which have epidemiologic and laboratory characteristics that enable their subdivision (64). Variola major has a case fatality ratio of from 25 to 40 percent in unvaccinated individuals, and some 5 percent of patients have severe hemorrhagic smallpox and die before characteristic pock-like lesions appear on the skin (65). The virus strain of Variola major grows best at 35°C and does not grow at 39°C. Variola minor has a case fatality ratio of approximately 1 percent, and rarely produces hemorrhagic disease (6). Variola minor virus grows well at both 35° and 39°C. There is probably a variety of strains intermediate between Indian Variola major and Brazilian Variola minor (alastrim). In Africa and Indonesia, case fatality ratios in unvaccinated individuals are often approximately 10 to 15 percent, and there is evidence that strains from these areas have growth characteristics intermediate between Variola major and minor. These strains breed true; individuals with Variola major do not give rise to cases of Variola minor and vice versa.

The residual immunity from vaccination may attenuate smallpox even if the immunity is not sufficient to prevent infection. Patients with attenuated cases generally have fewer lesions on the skin and mucous membranes, and a much reduced risk of death (66, 67). Patients with vaccine-attenuated smallpox generally have severe enough prodromal symptoms so that they feel ill and are recognized as being ill, even though the diagnosis may not be obvious. When such mild cases go unrecognized, they may help spread the disease because they are not isolated, and are not incapacitated enough to voluntarily restrict their own movements. On the other hand, because of the small number of lesions, mild cases are not efficient vectors of smallpox (66, 67).

Epidemiologic evidence indicates that smallpox is clearly less infectious than measles or influenza, and probably less so than most of the childhood viral infectious diseases (68). The mean age at attack in endemic countries is generally higher than for measles. Smaller populations can maintain transmission of the disease than are required for measles and influenza. The susceptible-exposure attack rates are lower than with measles and influenza. In West Africa several instances have been documented where smallpox was maintained for several months in tiny villages or large family compounds of fewer than 100 individuals (68). The disease spreads generally to identifiable close contacts of cases; family members, household members, physicians and nurses, and other individuals with prolonged face-to-face contact with the patients (66–70). These characteristics make smallpox susceptible to control by case investigation, contact identification and tracing, and vaccination of potentially exposed susceptibles. This concept of creating a ring of immunized individuals around each case has become the fundamental tool of smallpox control and elimination, especially when the disease is introduced or reintroduced into previously smallpox-free areas (4, 70, 71).

RISKS OF INTRODUCTION AND SPREAD OF SMALLPOX

The risk of introduction of smallpox into the United States must be very low since there have been no importations since 1949. The risk is probably decreasing as the geographic reservoir shrinks (see Fig. 1) and the annual incidence declines. In addition to this decrease in the reservoir, there has been a change in the sociologic distribution of the disease. In virtually all endemic nations smallpox is now similar to poliomyelitis and diphtheria in the United States; a disease of isolated rural villages and urban slums. Smallpox attacks those who cannot afford to be immunized, or who are not served by even minimal health services. Such people have very little likelihood of taking a jet airplane to the United States.

Once a patient with smallpox reaches this country, how many indigenous cases might result? Following an introduction into Texas in 1949, there were 8 cases and 1 death (72). In New York City in 1947, there were 12 cases and 2 deaths (73). These are fewer than the average number of cases and deaths in recent European experience. This is best explained by our better public health services, rather than by our vaccination status. Both the New York City and rural Texas populations were poorly immunized (72, 73). The New York City authorities felt it necessary to perform 6.3 million vaccinations during the outbreak to raise the city's immunity level. Pre-existing levels of immunity did not prevent importation, and did little to control spread.

Since 1950, there have been 49 documented importations of Variola major into Europe (including the Eastern bloc nations). Seventeen, or one third, did not cause indigenous spread. The remaining 32 importations caused a total of 745 cases and 115 deaths, or approximately 23 cases per outbreak (26). Over half of these patients acquired their infection in a hospital, either because they were patients on the same ward as a smallpox patient, or because they were members of the hospital staff.

For calculating the risk of smallpox to the general United States population, we estimate that only two of every three imports will cause spread, that 23 cases will occur in each outbreak, and that a death-to-case ratio of one-third will result in eight deaths per outbreak. Alert surveillance of suspect smallpox cases and prompt patient isolation and vaccination of contacts could reduce this toll substantially.

RISKS VERSUS BENEFITS OF ROUTINE INFANT VACCINATION

The United States currently records at least seven deaths per year attributable to smallpox vaccination. Some four of these seven fatalities are caused by postvaccinial encephalitis, one by eczema vaccinatum, and two by vaccinia necrosum (11). If we ignore the considerable morbidity associated with routine childhood vaccination and examine only mortality, we can predict that the United States will experience at least 210 vaccine-related deaths during the remaining 30 years of this century under the current vaccination. Two-sevenths of these deaths, specifically those caused by vaccinia necrosum, are either preventable or occur in patients who might die of their underlying disease even if not vaccinated. The postvaccinial encephalitis deaths are not preventable and can only be reduced by reducing the number of vaccinations performed. All recent deaths from eczema vaccinatum have been in contacts rather than vaccinees, and could not have been prevented by screening of vaccinees alone (11).

The logical means of reducing the number of vaccination complications is elimination of routine childhood vaccination. We would then vaccinate only high-risk individuals. Adult primary vaccination would then be common in military inductees, travelers to smallpox endemic areas, and new entrants into the health professions. With a risk of death of three per million adult primaries, and a maximum of 1.4 million adult primaries per year, there would be approximately four deaths per year (27). Since, at present, most of the United States population has received at least one vaccination, in the near future most high-risk individuals will continue to be revaccinees with little risk of death. Taking this into account, a maximum of 60 deaths from vaccination of the high-risk adult groups might occur in the next 30 years (27). With 210 deaths expected under the current policy, we might save at least 150 lives between now and the end of the century by elimination of childhood vaccination.

A proportion of the 150 fatalities avoided by discontinuing routine childhood vaccination might be counterbalanced by deaths attributable to smallpox itself. The number of importations of smallpox and the resulting number of cases per importation outbreak will determine the number of such cases. We believe that the risk of importation has diminished considerably because of the geographical and numerical diminution of the reservoir of smallpox. As we have already explained (see above), a maximum of eight deaths might be expected in each outbreak, but only two out of each three importations would probably cause an outbreak. With 16 deaths for each three importations, nearly one importation each year would be required to overweigh the 150 vaccinia-related deaths avoided by dropping routine childhood vaccination.

The estimate of the number of high-risk adult vaccinations performed annually is critical for this analysis. We assume that only travelers to endemic continents require vaccination. Further reduction of the number of vaccinations necessary for travel should occur when Asia, Africa, and South America become free of smallpox. If foreign quarantine policies demand vaccination of travelers to Western Europe, our estimate is unrealistically low. Our estimate of numbers of military recruits is based on recent figures. Military and political situations may change so that this figure may rise or fall. The United States military has not recorded a fatality caused by smallpox vaccination since World War II, however, so that recruits may not account for any fatalities even though we have included them with other adults. Our estimate of the number of new health workers per annum includes all medical, paramedical, and hospital workers, not only physicians and nurses. At present, many practical nurses, laboratory workers, and other paramedical personnel are not routinely vaccinated on entry into training. While this is unfortunate from an epidemiologic viewpoint, it means that our estimated number of vaccinations and deaths in this group is unrealistically high.

MAJOR OBJECTIONS TO ELIMINATION OF ROUTINE CHILDHOOD VACCINATION

Considerable debate has underscored several objections to changing the current policy of routine childhood vaccination. First, neither our estimate of the death rate in adult primary vaccinations nor our estimate of the number of adult primary vaccinations to be performed annually is based upon firm United States data. Either of these estimates may be higher or lower than reality; if both are too low, then the risk of vaccinating high-risk adults may be greater than the risk of routinely vaccinating children. One important unknown is the future policy for vaccinating travelers to nonendemic continental land masses.

Second, a lower population immunity will lower our defense against biological warfare using smallpox virus. Smallpox can be lyophilized and is contracted by the inhalation route, making it useful for biological warfare. The current policy of routine childhood vaccination does not fully protect us from biological warfare. Were we attacked today, we could expect widespread disease. If the fear of biological warfare influences our immunization policy for smallpox, then it will be the only disease for which this factor has been considered in decision making. There are several other agents potentially useful for biological warfare, but we do not routinely immunize our population against them.

Third, several authorities worry that discrediting smallpox vaccination will lead to public doubt about other important immunizing agents. The United States is already insufficiently protected against poliomyelitis. Measles eradication, which should have been rapidly achieved with the available safe and effective vaccines, has not yet taken place. It would be tragic if the public assumes that all vaccines are either unnecessary or unsafe simply because the risk of vaccinia is higher than the risk of smallpox in the United States at the current time. Scientific decision making should not be affected by vague fears of public misinterpretation; rather, good health education should be employed to help the public comprehend such decisions.

Fourth, many public health workers remember the 1947 New York City outbreak, when mass vaccination efforts disrupted medical and public health work for many days (73). An importation of smallpox might cause panic, and during injudicious mass vaccination insufficient attention given to the contraindications to vaccination might cause many vaccinia-related deaths. We believe that the techniques of contact tracing and selective ring vaccination should be used to control importations. Such methods should keep the number of vaccinations performed to a minimum, and would not place undue demands on the funds or personnel at the disposal of medical and public health authorities. In the unlikely event that a mass vaccination campaign would have to be mounted, health services should be much less disrupted than they were in 1947. Vaccine production techniques, especially lyophilization, enable vaccine to be stockpiled and shipped without refrigeration. Vaccine supply therefore should not be a problem. Jet injector vaccination technology enables untrained personnel to give thousands of effective vaccinations per day (74). Vaccination in a mass campaign need not be performed by skilled personnel, who could perform their usual duties, trace smallpox contacts, or screen prospective vaccinees for vaccination contraindications.

Fifth, there are emotional as well as logical considerations in framing vaccination policy. The general public and medical profession have lived with endemic vaccinia complications. If all of the vaccinia-related deaths took place in one town in one month, the public might be excited, but in the present situation it does not feel vaccinia is dangerous. One or two cases of smallpox, and certainly half a dozen fatalities from smallpox, would create considerable public emotion and outcry. If such an incident followed shortly after cessation of childhood vaccination, the public and many members of medical and public health professions might mistakenly view the policy change and the smallpox outbreak as cause and effect. Death from vaccination should be as repugnant as death from smallpox; emotional judgements should not color scientific decisions.

Although the current policy has not produced solid immunity throughout the population, many people believe that there may have been importations of smallpox in recent years which were not detected because secondary cases did not occur. We believe this is extremely unlikely. Patients with smallpox, even vaccine-modified smallpox, are extremely ill and come to medical attention. If their illness is so mild, or so modified by previous vaccination that they do not seek medical help, they are unlikely to be shedding enough virus to spread the disease. With at least 10 and perhaps 50 percent of the American population incompletely immunized against smallpox, the chance of an unrecognized smallpox patient having close contact only with fully protected individuals is extremely low.

The evaluation of vaccination policies for smallpox, as well as for other communicable diseases, should be a continuous process. The factors which influence policy decisions change from time to time and in place to place. Major changes in the world distribution of smallpox, development of new vaccines, and development of treatment methods for vaccination complications may further change the risks of smallpox and vaccination.

CONCLUSIONS

At present, smallpox is limited to the Indo-Pakistani subcontinent, Brazil, Indonesia, and a few subsaharan African countries. We believe that by the mid-1970's it may be limited to Ethiopia and the Indian subcontinent. Smallpox is a dramatic and clinically overt disease, which spreads by the respiratory route to close identifiable contacts. There is no known nonhuman reservoir of the virus. The majority of smallpox importations into nonendemic Western European countries in the past two decades have resulted in either no spread of smallpox or small outbreaks which were readily contained. Importations into the United States could probably be contained by outbreak investigation and selective vaccination of contacts.

The herd immunity of the United States population is difficult to assess. The immunity caused by vaccination is of uncertain duration. The risk of acquiring smallpox is close to zero for three years after vaccination, but by 20 years there is little residual immunity. Only 7 percent of the United States population is vaccinated each year, and probably half of the population has not received a vaccination for 20 years. The immunization of travelers and surveillance of immigrants' vaccination status by the foreign quarantine program are responsible for freedom from smallpox in this country, not the population's vaccination status.

Smallpox vaccination carries definable risks. In the United States at least seven deaths each year are attributable to vaccination. The fatality rate is approximately one per million primary vaccinations. Morbidity is considerable. At least 210 deaths might be expected in the next 30 years from the current policy of routine childhood vaccination. Vaccination of high risk groups, including military personnel, travelers to endemic areas, and health workers, might reduce the number of deaths in the next 30 to 60 years. Thus, the present policy of routine childhood vaccination will be responsible for approximately 150 unnecessary deaths in the remainder of this century.

The risk of reintroduction of smallpox is too low to expect as many as 150 smallpox deaths in the next three decades. Therefore, in the United States at this time, the risk of routine childhood smallpox vaccination is greater than its benefits.

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USE OF PHYSICIANS' ASSISTANTS IN THE DELIVERY OF MEDICAL CARE

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This review will not cover the education, training, and use of nurses, dietitians, physiotherapists, clinical psychologists, technicians of all kinds and occupational therapists in their traditional roles. It will cover only those developments in which nurses are assuming functions traditionally reserved in our culture for doctors, or where new programs are training various types of assistants to perform functions traditionally reserved for doctors.

The medical profession, the colleges and high schools, the medical schools and medical centers, the lay public and governmental funding agencies have all accepted the premise that doctors are, and will be for a number of years, in short supply. The nation is ready and able to purchase a larger quantity of personal health services. These services should be of high quality and low unit cost. Two types of programs are being developed:

1. Upgrade the education and functions of the nurse so that under the direction of a doctor she can carry out functions currently being performed only by doctors.
2. Train new categories of persons who can become members of the physician's health team.

Both these programs envision that the new personnel will engage in what is currently the diagnosis and practice of medicine. The pediatric nurse practitioner, the coronary care nurse specialist, the inhalation therapist, the physician's assistant are being taught to carry out functions which in the past have been performed only by physicians. In a few states, these functions have been legalized by changes in the medical practice art. In a majority of states, they are functioning with the tacit approval of the medical profession, because no one has a better solution to the needs of the public.

UPGRADE THE FUNCTION OF THE NURSE

Nurse Clinics

Dr. C. E. Lewis and Barbara Resnik, R.N., M.P.H., described in 1967 their experience with Nurse Clinics and progressive ambulatory patient care (1). Patients included in the study had hypertensive cardiovascular disease, arteriosclerotic heart disease, exogenous obesity, psychophysiologic reactions, and arthritis. The Nurse Clinic operated in the medical outpatient area. The nurse had her own office and scheduled her patients' appointments. The Nurse Clinic was listed among those clinics held by the Department of Medicine. The experimental group of patients (those seen in the Nurse Clinic) accepted the nurse as a primary source of care. The quality of health care in the Nurse Clinic was compared with that in the conventional medical clinic. There was increased adherence to appointment schedules and better utilization of time, as demonstrated by time and motion studies. The overall cost of the program on a dollar basis was less. The quality of care and patient satisfaction with care were higher in the experimental group.

THE NURSE IN AMBULATORY PATIENT CARE

At the Massachusetts General Hospital, the medical and pediatric services have initiated programs in conjunction with the nursing service where the nurse undertakes preventive and supervisory care of ambulatory patients (2). In the pediatric area, broad mandate is given to promote preventive care in communicable disease and nutrition, to analyze family interaction, to detect physical, mental, and emotional handicaps and to work in accident prevention and family planning. The nurse participates in the care of adult patients with chronic disease. She is responsible for maintenance of patient participation in medical treatment over long periods, prevention and early recognition of complications of the disease or its treatment, and management of the ordinary emotional adjustment problems.

PEDIATRIC NURSE PRACTITIONER

In the mid-1960s, Dr. Henry Silver (3), at the University of Colorado, developed the pediatric nurse practitioner department as a joint venture of the Department of Pediatrics and the School of Nursing. After a four-month training period at the Medical Center, the pediatric nurse practitioner functions in the office of pediatricians in private practice and in field stations in low income and rural areas where she is readily accessible to people. In the field stations, the nurses have office hours suited to the particular population groups in the adjacent areas.

> The nurses provide total well-child care and make significant contributions in the supervision of infants by giving mothers instruction regarding many items of child care, including formula preparation, infant feeding, bathing, toilet training and accident prevention, as well as counseling about a number of minor physical and psychological problems. The nurses' services are particularly meaningful in counseling young, inexperienced mothers. Routine checkups of infants and older children, developmental testing, various screening procedures and tests, routine immunizations, complete physical examination when indicated, as well as the management of a number of minor disorders can all be carried out by the nurse. In caring for these patients, the nurses employ their nursing talents

> to the fullest. At the same time the specialized skills of the physician are more effectively and wisely employed.
>
> The child who is ill also has a complete evaluation, including a comprehensive history and physical examination. With a plan of management previously agreed upon, the nurses may handle the problem themselves or refer the child for immediate attention elsewhere. Special emphasis is placed on the importance of followup continuity of care.

Dr. Silver emphasizes that the educational experience the nurse receives is important.

> We feel that the type of educational experience the nurses receive is important. In order to perform the increased duties and assume the responsibilities of pediatric nurse practitioners, the nurses entering our program must undergo a significant role reorientation so that they can function effectively in their new positions. On-the-job training does not permit the nurses to make the transfer from their previous professional identification to the new one. The physicians must demonstrate to the nurses that they and the nurses can be comfortable when the nurses are carrying out functions that were formerly within the province of the physician. Physicians must participate in authenticating the nurses' new role.

NURSE MIDWIVES

In 1959 Dr. Louis Hellman (4, 5), at New York State University-Kings County Medical Center, began to train and use midwives. Five years later, 86 persons had gone through the program and received certification as nurse midwives. All these nurses had R.N. degrees, and some the Bachelor or Masters degree. Only 13 of the graduates engaged in the practice of nursing midwifery. The majority of the graduates were employed as teachers, missionaries, or in the Public Health Service.

The nurse midwife works on the obstetrical floor. The medical resident has the responsibility for the initial examination of the patient and for the assignment of the patient to the midwifery service. The midwife then gives total care to her patient. This means not only conduct of labor and delivery but administration of total nursing care. The midwife can administer analgesia within limits, perform pudendal blocks at delivery, and repair a median episiotomy.

CLINICAL NURSING SPECIALISTS

The establishment of coronary care, respiratory, and renal dialysis units has led to a broadening of the nurse's role in the delivery of personal health services. Other physicians on the staff usually expect the director of the unit to be responsible for their patients when in the specialized area of care. The director of the unit usually gives a combination of didactic and on-the-job training to the nurses. He will eventually delegate to his nurses many of the responsibilities which are reserved for the doctor in other areas of the hospital. Gradually, but surely, the nurse specialist is being created.

NEW COMPONENTS OF THE PHYSICIAN'S HEALTH TEAM

TYPE A ASSISTANTS

See reports of Board on Medicine (6) and Committee of Association of American Medical Colleges (7).

Physician's assistant.—In the mid-1960s, doctors began to appreciate that they needed help from assistants who were selected by doctors, educated by doctors, and paid by doctors. In 1964, the Duke Medical Center began to develop the man component of a projected man-machine clinical support system for the doctor giving personal health services. The clinical faculty of the medical school select as students mature high school graduates who wish to work with doctors in delivering personal health services at the community level. The students have previously worked in the health field and have found satisfaction in their work. They are of good intelligence, have a rapid rate of learning, and most of them are men who have served in the medical corps of one of the armed services.

The clinical faculty gives these students, whom they refer to as "physician's assistants" (PAs), a two-year course leading to a certificate from the Duke Medical Center. The first nine months of the course are primarily didactic. Six weeks are spent in lectures in medical terminology, medical history and ethics, and basic laboratory procedures. Six months are spent in an integrated series of lectures, arranged by organ systems and covering anatomy, physiology, disease states and principles of therapy. During the last half of this period, instruction in history taking and physical examination is begun. The last six weeks are spent in an introduction to radiology and electrocardiography, plus an introduction to the public health system.

The remaining 15 months are spent in a series of clinical rotations. An inpatient ward rotation, an outpatient rotation including emergency room experience, and a rotation in the office of a community practitioner are all required. The remainder are arranged to fit the interests and career choice of the trainee.

The PA learns to take a history, to do a physical examination, to record the findings, and to present them in an organized way to the physician. He is taught to do technical procedures and to instruct patients. He is also able to carry out emergency cardiorespiratory support procedures, intubations, venipunctures, arterial punctures, minor suturing, and many other tasks. He knows how to approach the patient, to gather data and to organize the data so as to make the physician's job easier, and how to help the patient follow instructions regarding treatment.

In practice settings, the PA's duties vary with the activities of the physician. In a typical internist's setting, he performs and records histories and physical examinations, helps with patient instructions, records laboratory data, completes forms, follows patients in the hospital, et cetera. In a general practice setting, he may also suture minor lacerations, change dressings and remove casts. In an endocrinologist's setting he may assume responsibility for arranging and performing complex tests involving precise timing and precise methods of sample collection. Any duty which the physician does repetitively might be assigned to a well-trained assistant. The assistant works long hours, usually 50 or more a week, and his beginning salary is about $10,000 per year (8).

Child health associate.—On July 14, 1969, Dr. Silver (3) began a program to educate an entirely new type of health worker who would be associated with physicians in giving diagnostic, preventive, and therapeutic services to patients. The graduate of the Child Health Associate Program will enter practice five years after he graduates from high school.

> Students enrolling in the Child Health Associate Program have completed two or more years of work in an undergraduate college or university. This is followed by a two-year course of instruction at our Medical Center and a one-year internship. The curriculum for the two years at the Medical Center consists of a chronologic development of pediatrics from antenatal life to adolescence—with basic science, psychosocial and clinical training and experience emphasized at each developmental period. Students will receive a Bachelor of Arts degree upon completion of the first two years at the Medical Center. The internship year follows. This internship will be similar to a straight internship in pediatrics but with emphasis on clinical experience in the outpatient clinics, in the offices of private physicians, and in community facilities. During this year, relatively little time will be spent on the hospital wards.
>
> The graduate will limit themselves to the care of well children and will not be concerned in providing care for seriously ill children. Since hospitalization of pediatric patients is generally limited to those with severe illness or complex problems, hospital care (except for some aspects of routine newborn nursery care) would automatically be considered outside the practice of the child health associate. Within the limits established, the child health associate will be qualified to diagnose, counsel and prescribe in both health and disease.
>
> It is expected that graduates of the Child Health Associate Program will practice in two main areas: (a) as associates and employees of physicians (both pediatricians and generalists) in their private offices or (b) under the direct supervision of individual physicians in various public health departments. At all times, child health associates will function under the personal and direct supervision of a licensed physician.

Broadly trained physician's assistants produced by medical centers have not been available in the United States. They differ from other paramedical personnel in that they are taught by doctors to perform functions which doctors traditionally perform. Community colleges and colleges of allied health professions which do not have a large faculty of physicians cannot educate these assistants. Most doctors, not having worked with well-trained assistants, do not appreciate how useful they can be in the delivery of personal health services.

The Board on Medicine of the National Academy of Sciences has published a report (6) urging the development of these broadly-trained assistants which they elect to call "Type A." They point out that the assistant is a dependent portion of the doctor's health team and that his independence must lie in the opportunity to evolve into a doctor. The Board comments as follows:

> As far as can be visualized in the future, it is felt that these assistants should perform as members of a health team, under the general supervision and authority of a physician or group of physicians. The provision that these assistants shall perform in a dependent relationship with physicians in fact expands the range of functions which are or may come to be within their sphere of competence. Of the various independent practitioners in the health field, only the physician is authorized to perform over the full spectrum of medical care. The more narrowly defined sphere of activity necessary for other independent practitioners is likely to influence strongly, if not firmly dictate, the limit of their functions and development—if for no other reason than that it bears so heavily on the spectrum of problems which will be presented to them. By contrast, assisting with the variety of problems which confront physicians over a period of time provides an opportunity for continuous learning and encourages the development of new skills which would justify a rising ceiling on the activities of the conscientious assistants.
>
> The functions performed by such assistants should be within the areas of medical practice in which the responsible physician or physicians are clearly competent. For example, it would be inappropriate for a surgeon's assistant to perform a preoperative cardiac evaluation unless the surgeon is competent to review his work critically.
>
> Some assistants may wish to become independent and they may do so by obtaining an M.D. degree. On the basis of performance and equivalency examinations they will be able to demonstrate that they have mastered many of the functions and concepts normally taught medical students in their clinical years. Type A assistants should be able to go through medical school in less than the usual four academic years.

The Council of Academic Societies has approved and forwarded to the Executive Council of the Association of American Medical Colleges a report of its ad hoc committee on physician's assistant programs (7). The report deals primarily with the broadly trained assistant. This Council uses the same terminology as the Board on Medicine, calling these Type A assistants. They make the following recommendations:

> A. The AAMC should demonstrate leadership in the definition of the role and function of these new categories of health care personnel, in setting educational standards for programs producing them, and in considering the additional problems raised in the preamble.
>
> B. The AAMC should seek the counsel and the cooperation of other interested organizations and agencies as it moves ahead in the above task.
>
> C. The AAMC should work toward an accrediting agency as a means of effective accreditation and periodic review of programs producing such personnel. A joint liaison committee with the AMA, similar to the Joint Liaison Committee for Medical Education, is one suggested mechanism.

The report gives, in considerable detail, guidelines for educational programs for Type A assistants.

TYPE B ASSISTANTS

See reports of Board on Medicine (6) and committee of Association of American Medical Colleges (7).

Inhalation therapists.—In the last 10 years, doctors who carry out highly specialized tasks have developed personnel to help them. These persons in the area of the specialty have a degree of skill in excess of that possessed by most physicians outside the specialty. The area of inhalation therapy is a good example. The Board of Schools of Inhalation Therapy is organized under the auspices of the Council on Medical Education of the American Medical Association and is sponsored by the American Society of Anesthesiologists, the American College of Chest Physicians, and the American Association of Inhalation Therapists. In 1962, the Council on Medical Education of the AMA established a mechanism for approving schools. Two recent textbooks of Inhalation Therapy describe the organization and staffing of the schools (9, 10).

The inhalation therapist is following the pattern laid down by other health professionals. The course is lengthening, the educational requirements, before admission to the school, are increasing. Inhalation therapists are in short supply, and inhalation technicians are being trained to assist the therapists.

As the educational background is broadened, the leaders in the field have begun to question the desirability of being quite so specialized. They point out the overlap between the pulmonary and cardiovascular areas and are considering the desirability of educating a broader-based inhalation-cardiovascular therapist.

The inhalation therapist operates under the supervision of a physician, usually a chest physician, an anesthesiologist, or an internist. The exact legal and moral responsibilities of the medical director of the inhalation therapy unit and its staff have not been clarified.

Orthopedic assistants.—In 1970 the American Medical Association approved the essentials for accrediting educational programs for orthopedic assistants (11). Applications for accreditation of an educational program for orthopedic assistants should be made to the Department of Allied Medical Professions and Services, American Medical Association, 535 North Dearborn Street, Chicago.

TYPE C ASSISTANTS

See reports of Board on Medicine (6) and committee of Association of American Medical Colleges (7).

Many doctors are training helpers with a combination of a short didactic course and a year of on-the-job training. The MEDEX program, developed under the auspices of the medical school of the University of Washington and the Research Foundation of the Washington State Medical Association has taken this direction (12).

Men who have been successful in the medical corps of the Navy, Army, Air Force, or Coast Guard are given a 15-month program consisting of three months of university training and 12 months of preceptorship with a doctor who has agreed to employ him at the end of the preceptorship. During the preceptorship, there are 10 three-day weekend continuing education seminars. Tasks which the new professional can perform include screening patients to be seen by the physician, history taking, performing parts of physical examinations, the application and removal of casts, assisting at surgery, suturing minor lacerations, taking roentgenograms and performing laboratory tests during nonoffice hours, assuming certain administrative responsibilities, and being available to provide the physician with assistance any day of the week and any hour of the night.

LEGAL CONSIDERATIONS

Most medical practice acts state that the physician can diagnose and treat patients, with no provisions to enable the physician to delegate tasks. Arizona, Colorado, Kansas and Oklahoma have written in exceptions which allow the delegation of tasks to assistants under the control and supervision of a licensed physician.

A document entitled "Model Legislation Project for Physician's Assistants" has been prepared by the Department of Community Health Sciences of Duke University Medical School (13).

The reports of the Board on Medicine and the Committee of the Association of American Medical Colleges and the Duke document on model legislation emphasize that the Type A assistant is unique in the medical field and that the extent of his potential has not been determined. They recommend that legal authorization not be effected by licensure but, rather, by authorization built around a system of registration which would permit qualified physicians to employ assistants if such assistants have completed an approved program or have otherwise established their qualifications to perform the duties proposed for them. The basic objective is to provide protection to the public from unqualified personnel without imposing the rigid definitions characteristic of licensing legislation.

The Colorado Child Health Associate Law (3) "includes provisions so that no more than one child health associate will be employed at any one time by any one physician. Except in an emergency, the child health associate will practice only in the professional office of the physician and only when he is personally available. The associate will render services outside the office only in the presence of the physician, or in caring for patients pursuant to the directions of the physician in relation to particular patients. The child health associate will be permitted to prescribe by prescription, but will prescribe only such drugs as shall be approved specifically by the State Board of Medical Examiners for prescription by the child health associate."

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ALPHA$_1$ ANTITRYPSIN DEFICIENCY AND PULMONARY EMPHYSEMA

7018

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The association of familial alpha$_1$ antitrypsin deficiency and pulmonary emphysema was first recognized by Laurell & Eriksson (1). This observation was particularly significant because it identified an important relationship between a biochemical abnormality and a common but poorly understood type of lung injury, a relationship that may represent the first documented disease resulting from a deficiency of proteinase inhibitors. In this review we have summarized the available information regarding the characteristics of serum proteinase inhibitors, the incidence and type of pulmonary disease associated with familial alpha$_1$ antitrypsin deficiency, and the evidence that pulmonary tissue injury may be related to the deficiency of these inhibitors.

PROTEINASE INHIBITORS

Many naturally occurring proteinase inhibitors in plants and animals have been identified and recently reviewed (2). In some instances such as the soybean, the function of the trypsin inhibitor appears to be incidental; in others, such as the nematode, *Ascaris lumbricoides,* the inhibitor properties may be essential for survival in the digestive tract of the host (3). Several species-specific proteinase inhibitors have been isolated from various organs and tissues. In man, the best evidence currently relates to the inhibitor in serum, pancreatic juice (4), colostrum (5), and urine (6). Additional evidence suggests the presence of proteinase inhibitors in the nasal and bronchial mucus, submandibular glands, seminal vesicles, endometrium, platelets, placenta, and amniotic fluid (2). This review will deal primarily with the proteinase inhibitors in serum, although tissue concentrations and specific organ or tissue localization of proteinase inhibitors may be of major significance.

Several protein fractions in serum identified by their electrophoretic migration have been demonstrated to be proteinase inhibitors (2): (*a*) postalbumin fraction; (*b*) α1-globulin fraction; (*c*) interalpha fraction; and (*d*) an α2-globulin fraction. Schultze (7) demonstrated that the major inhibitor for trypsin was located in the α1-globulin fraction and suggested this be called α1-antitrypsin. Subsequently, it became clear that this protein also inhibits the activity of elastase (8, 9), collagenase (10), proteolytic enzymes from human leukocytes (11), thrombin, and plasmin (12). This led to the suggestion by Gans & Tan that the term α1-antiproteinase replace the term α1-antitrypsin (12). This protein has a sedimentation constant of 3.85 and a calculated molecular weight of approximately 60,000.

Genetic control of the blood levels was established by the recognition of familial α1-globulin deficiency (1). Initial evidence suggested a simple autosomal recessive mode of inheritance. Homozygous-deficient individuals were found to have α1-antitrypsin levels that were about 10 percent of the levels in the normal population, and the small quantity of antitrypsin present migrated more slowly than normal in an electrophoretic field. Heterozygous-deficient individuals had about 55 percent of the normal antitrypsin levels (13). Fagerhol & Laurell then demonstrated multiple distinct electrophoretic variants of the α1-globulin occurring spontaneously in the population and suggested that these variants were determined by multiple codominant alleles. Since these proteins are protease inhibitors, they designated them as "Pi" types (14). Subsequent studies by acid starch gel electrophoresis demonstrated seven distinct Pi types in Swedish and Norwegian population studies (15). These Pi types, given symbols in order of decreasing migration rates, are Pi^F, Pi^I, Pi^M, Pi^S, Pi^V, Pi^X, and Pi^Z. Fagerhol then used antigen-antibody crossed electrophoresis in the quantitation and immunologic identification of protein separated by starch gel electrophoresis, adding further support to the concept of multiple Pi types determined by codominant alleles (16). The phenotype MM is the most common in the population and is associated with a normal level of α1-antitrypsin. Pi types MS, SS, MZ, and ZZ are the most common ones associated with decreased levels of the protein in the serum. In a study of 2830 Norwegians, Fagerhol (17) described the prevalence of these Pi types: 2 ZZ, 4 SS, 4 SZ, 81 MZ, and 116 MS. At present, only the homozygous-deficient, ZZ phenotype has been clearly implicated in association with chronic obstructive pulmonary disease, but most studies have not included Pi types in characterization of the protein.

Several studies have reported antitrypsin or α1-globulin levels in selected populations in the United States (18–23). The techniques of assay have included enzymatic inhibition tests, immunodiffusion or immunoelectrophoresis, and crossed antigen-antibody electrophoresis. No large-scale study of the general population has been reported to document the prevalence of various antitrypsin levels in the blood, and the incidence of various phenotypes has not been established in this country. Studies of pulmonary disease populations indicate that approximately 1 to 2 percent of patients with chronic obstructive pulmonary disease have the severe α1-antitrypsin deficiency (18, 19) likely to represent the Pi ZZ type, although some authors (20) have reported as high as 10 percent of such patients to be severely antitrypsin-deficient.

Although the major mechanism of control of the protein levels is undoubtedly genetic, several other factors are apparently significant. Ganrot & Bjerre (24) found increasing levels of α1-antitrypsin throughout pregnancy, which returned to normal shortly after parturition. They, however, found no other relationship of blood levels with age or sex. The administration of estrogens and progesterone may be associated with increased levels (25–27). Injection of typhoid vaccine produced marked increases in the α1-antitrypsin in individuals with normal blood levels, but an attenuated response was seen in those with the heterozygous-deficient state, and no increase was seen in the homozygous-deficient individual (28). Small increases in α1-globulin in some patients with diabetes mellitus (29) may be related to recurrent infection which is well known to increase the blood levels (30). In spite of these and other conditions associated with altered blood levels (2), the site of production, distribution, and degradation of the protein has not been established. Nakino & Reed (31) recently determined the distribution and disappearance of exogenous α1-antitrypsin given as whole plasma in three patients with homozygous α1-antitrypsin deficiency. They concluded that the protein was distributed in the extracellular fluid compartment and had a half-life of five to six days in these patients. Kueppers & Fallat (32) studied the half-life of purified I^{125}-labeled alpha$_1$ antitrypsin in normal and in heterozygous- and homozygous-deficient individuals. The half-life in normal individuals was three days, and was similar in the other two groups, suggesting that the disappearance of the protein due to degradation or excretion is normal in deficient subjects and that these individuals have a defect in synthesis of the protein.

HOMOZYGOUS ALPHA$_1$ ANTITRYPSIN DEFICIENCY AND PULMONARY DISEASE

A significant association of obstructive pulmonary disease with homozygous deficiency of α1-antitrypsin has been amply documented by Eriksson (13) and confirmed by others (8, 20, 21, 23, 33–42). Only an estimate of the precise frequency with which these conditions coexist is possible, however, and the mechanism causing their coexistence remains speculative.

Eriksson, in an extensive hospital screening program (13), identified 33 hospital patients with homozygous-deficient serum antitrypsin levels. Of these, 23 had chronic obstructive pulmonary disease. Of the remaining 10 subjects, 6 were not yet 40 years of age and might well develop lung disease later in life. Similarly, other laboratories have reported symptomatic lung disease in most, but not all subjects with homozygous antitrypsin deficiency. Most of the subjects have been identified because of the presence of disease or through family studies, however, and a large number of prospectively identified subjects with antitrypsin deficiency is not yet available for study.

The proportion of chronic obstructive pulmonary disease (COPD) patients accounted for by this protein deficiency has varied widely in the several reports dealing with this question. The variation appears to relate to selection of the patient population under study. When broad criteria are used and all patients with COPD are considered, roughly 1 in 100 have α1-antitrypsin deficiency (18, 19). When only emphysema patients are studied, to the exclusion of subjects with predominant chronic bronchitis, and when selected young patients, or those with a family history of emphysema are studied, the frequency of antitrypsin deficiency has been reported as high as 25 percent (21, 37).

The characteristics of the lung disease itself were first studied by Eriksson (13), and his observations have been verified and amplified since by independent laboratories (8, 21, 39). Although the protein deficiency affects males and females equally, the lung disease may involve males with somewhat greater regularity (13). Smoking, occupational exposure to lung irritants, or bronchopulmonary infections appear not to be prerequisites for the development of lung disease. Symptoms most often appear in the third or fourth decade of life, which is earlier than the onset reported by most other patients with COPD. Eriksson reported a wide spectrum of chronic obstructive bronchopulmonary conditions in his series including bronchitis, emphysema, asthma, and bronchiectasis. He commented, however, upon a greater-than-usual frequency of emphysema without pre-existing chronic bronchitis.

Other observers have been more strongly impressed by the uniformity of clinical appearance of these patients (8, 39, 43). In one series the clinical presentation almost invariably suggested emphysema without chronic bronchitis (39).

Physical examination of a typical patient (13, 39) reveals evidence of weight loss, fixed chest hyperinflation with hyper-resonance, distant or absent breath sounds at the lung bases, and often dry crackling inspiratory rales at the lung bases. Chest radiographs confirm the impression of hyperinflation and demonstrate a bilateral decrease in vascular markings to the lower lung fields, with normal or even increased markings, tapering normally, in the upper zones (39, 44–46). Lung scans have likewise shown a characteristic bilateral decrease in lower zone perfusion (46) and xenon ventilation and perfusion studies have demonstrated corresponding decreases in both parameters in the lower zones (43).

Spirometry (13, 39) reveals marked slowing of expiratory flow rates and reduced vital capacity, poorly responsive to bronchodilators. High total lung capacity, with high residual volume and functional residual capacity, have been shown by inert gas dilution and plethysmographic studies (13, 47). Airways resistance has been shown to be increased, while elastic recoil is severely reduced (47). The slowing of expiratory flow seen in these patients is probably due to small airway collapse related to loss of the radial traction support normally provided by intact alveolar walls. Intrinsic airways disease may be absent, particularly early in the disease.

Pulmonary gas transport studies (39) have shown increased physiologic dead space, with high alveolo-arterial oxygen tension gradients while breathing room air. Arterial hypoxemia without carbon dioxide retention is usual. The steady-state carbon monoxide diffusing capacity is characteristically reduced.

Although peripheral edema and hepatic engorgement is not common until late in the disease, electrocardiographic evidence of right atrial and right ventricular hypertrophy are common (48). Hemodynamic measurements have confirmed the anticipated increase in pulmonary artery pressure and pulmonary vascular resistance, which decreased in response to vasodilators (48). Detailed angiography has demonstrated loss of small precapillary arterioles, more marked in lower than upper zones (48).

The clinical pattern of disease, then, is that described by Reid as characteristic of primary emphysema (49). The limited autopsy material as yet available (13, 19, 37, 38, 47, 50) suggests that the disease results in predominant panacinar emphysema. Bronchitis, bronchiectasis, or asthma may be present or even predominate in patients with homozygous αl-antitrypsin deficiency; however, these conditions are probably secondary or incidental to the basic disease process.

Long-term studies of patients with emphysema and antitrypsin deficiency are not yet available. Progression of the disease appears to be generally slow but variable. Although the effect of cigarettes on the disease course cannot be clearly stated at this time, incapacitating dyspnea appears to occur at a younger age in smokers than in nonsmokers (39).

No specific therapy is available. Attempts to replace blood levels of αl-antitrypsin are not feasible since the half-life of parenterally administered protein is less than six days (31, 32). Factors such as estrogens (25, 26, 27) and typhoid vaccine (28), which stimulate increased blood levels in normal individuals, have little effect on the protein level in the homozygous-deficient. At present, conventional therapy for chronic obstructive pulmonary disease is appropriate. Genetic counselling may be of preventive value, and avoidance of smoking and industrial air pollution is desirable.

Significance of Heterozygous Deficiency

The occurrence of homozygous alpha$_1$ antitrypsin deficiency in the general population is infrequent, and the lung disease associated with it accounts for a small proportion of all patients with obstructive lung disease. However, heterozygous deficiency has been reported to be present in as many as 5 to 14 percent (20, 22, 51, 52) of persons in the population. If persons of this genotype are predisposed to lung disease, this predisposition is of much greater numerical importance. Several authors have discussed this relationship.

No increased prevalence of disease in heterozygotes was evident to Eriksson in his initial report (13). He did not apply highly sensitive tests of pulmonary function in his large groups of familial heterozygotes, however.

Lieberman (20), using trypsin inhibitory capacity technique to identify heterozygotes, found 10 heterozygotes in a population of 66 hospitalized emphysysema patients. This frequency was significantly higher than the 3 of 47 found in a control population. In a subsequent paper (52), he has reported 21 heterozygotes out of a group of 126 patients with COPD, again differing from a control population with 13 heterozygotes identified out of a group of 278 factory employees. He concluded that heterozygosity predisposes to chronic obstructive pulmonary disease.

However, using the same serological techniques, Welch and associates (51) have reported 17 subjects with intermediate deficiency in a group of 146 chest clinic patients. This frequency was not significantly different from the 3 of 51 seen in their control blood donor population. Similarly, Larson and associates (53) have reported a heterozygote frequency of 13 out of 163 in a group of COPD patients, and 18 of 118 in control subjects. These data do not support the hypothesis that intermediate deficiency is important as a cause of lung disease.

Recently, Kueppers and associates (22) have demonstrated that crossed antigen-antibody electrophoresis may detect genetic heterozygosity in some subjects with normal serum concentrations of antitrypsin. They have shown evidence that as many as half of the subjects classed as heterozygous by their discriminating technique would be missed if only serum antitrypsin levels were measured. In addition, they have found 25 heterozygotes among 103 patients with obstructive lung disease, and only 14 among a control group of 100 subjects with a mean age of 36 years, and 8 among a control group of 88 subjects with a mean age of 80 years. This is a considerably higher frequency of MZ heterozygosity than the 3 percent reported in 2830 Norwegians studied by Fagerhol (17) with acid starch gel electrophoresis.

In addition, recent investigators have employed sensitive indicators of deranged pulmonary function in subjects with intermediate antitrypsin levels and found abnormalities in asymptomatic individuals. Stevens (47) found decreased elastic recoil and increased functional residual capacity in all subjects with intermediate antitrypsin levels; and Fallat (54) found abnormal patterns of pulmonary ventilation in such subjects using radioactive xenon. Age-matched control data and studies in familial heterozygotes are not yet available in sufficient quantity to permit these studies to be regarded as definitive.

On the other hand, prospective investigation of carefully preselected subjects from a community survey, and of obligate genetic heterozygotes from family studies, has been recently undertaken by Guenter and associates (55). These investigators, in a preliminary report, were unable to detect any relation between spirometric abnormalities and intermediate serum antitrypsin levels, even in the older age groups. In addition, they report no unusual frequency of disease or decreased survival in genetic heterozygotes. The current studies do not clarify whether serum capacity for inhibition of proteolytic enzymes (commonly measured as trypsin-inhibitory capacity) or serum levels of α1-globulin antitrypsin (commonly measured by immunodiffusion or immunoelectrophoresis, or starch gel electrophoresis) or genetic heterozygosity are most important in determining the predisposition to disease. Carefully selected control populations and similar criteria for determining the presence of pulmonary disease have not been employed in the various studies.

A reasonable hypothesis based on current data might suggest that intermediate antitrypsin levels or genetic heterozygosity predisposes to subtle pulmonary functional changes, but not to a greatly increased morbidity or mortality from lung disease. Further studies will be required to affirm or negate this hypothesis.

POSTULATED MECHANISM OF DISEASE PRODUCTION

It has not been clearly established that alpha$_1$ antitrypsin deficiency has a causal relationship to disease, although an association between severe deficiency and pulmonary emphysema has been satisfactorily demonstrated. An attractive pathogenetic mechanism has been hypothesized, however (11, 13). This hypothesis suggests that alpha$_1$ antitrypsin is essential to protect the lung against the destructive action of naturally occurring proteolytic enzymes. Possible sources of such proteases include bacteria, leukocytes, macrophages, and serum. Kueppers & Bearn (11) have partially tested this hypothesis by demonstrating the ability of alpha$_1$ antitrypsin to inhibit the action of leukocytic proteases, but not bacterial proteases, on fibrin. Pierce and associates (9) have suggested that the collagen and elastin network of the lung may be the site of injury in this disorder and have demonstrated reduced ability of the serum of antitrypsin-deficient subjects to inhibit the action of elastase and collagenase. Most recently, Lieberman and co-workers have demonstrated the ability of alpha$_1$ antitrypsin to inhibit leukocytic protease from purulent sputum, using a suspension of human lung as substrate (56).

ANTITRYPSIN DEFICIENCY AND EXTRAPULMONARY DISEASE

Eriksson's original series of subjects with homozygous alpha$_1$ antitrypsin deficiency, still the largest reported, did not demonstrate an unusual incidence of extrapulmonary disease (13). Peptic ulcer disease was observed in 30 percent of his patients, but this was no higher a frequency than that reported in an earlier series of patients with chronic obstructive pulmonary disease. Lieberman reported similar data (20).

A recent report by Sharp and associates (27) has linked liver cirrhosis in childhood with severe antitrypsin deficiency in ten subjects. Family studies were compatible with homozygous inheritance of an autosomal recessive trait in these children. This relationship has not been documented in other reports. An association between hereditary antitrypsin deficiency and cirrhosis has not been observed in adults. One autopsy report has described liver abnormalities in an adult with antitrypsin deficiency and emphysema (41), who also demonstrated the pathologic findings of necrotizing angiitis and glomerulonephritis.

Decreased serum trypsin inhibitor has been shown in 12 of 14 patients with respiratory distress syndrome, by Evans and associates (57). The deficiency was not demonstrated to be of genetic origin in these infants, and its role in the production of the lung disease remains speculative.

OTHER GENETIC FACTORS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

An awareness that genetic factors have an important role in the development of COPD has been evident in the clinical literature for many years. In spite of this awareness, a relatively distinctive clinical form of lung disease, with a predictable pattern of inheritance, escaped notice until the present decade. This review has summarized the available information concerning this disorder, the pulmonary emphysema associated with hereditary deficiency of alpha_1 antitrypsin. Recent evidence has stimulated speculation concerning other genetic factors which may be important in the etiology of bronchitis and emphysema. Larson and associates (53) have documented a distinct tendency to familial grouping in patients with COPD which is not related to antitrypsin deficiency. Further clarification of the long recognized but poorly understood role of heredity in lung disease can be anticipated.

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CLINICAL APPLICATION OF CONTINUOUS MONITORING OF RESPIRATORY FAILURE[1]

7019

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The management of respiratory failure has improved greatly in recent years. This has been due to some extent to an improved understanding of respiratory physiology, but the major reason lies in the application of this knowledge to patients, particularly in intensive care units. Why the creation of such specialized areas was so long delayed is a mystery, the need having been obvious for so many years. Historically, it is probably fair to say that coronary intensive care units were the first to achieve widespread acceptance. In these units continuous monitoring was relatively easy to achieve, requiring electrocardiography and, less often, the continuous recording of arterial and central venous pressures. Equipment had been standardized, was relatively uncomplicated, and could be understood readily by medical and paramedical personnel.

The patient in respiratory failure presents a more difficult challenge. His condition can deteriorate in seconds, and for reasons which are hard to understand graduates of medical and nursing schools continue to this day to be depressingly unaware of the measures needed to prevent and treat such deterioration. Respiratory depression and obstruction are poorly understood, and the necessity for adequate removal of CO_2 from and the addition of O_2 to blood in the lungs is often given little heed. Even in respiratory intensive care areas it is common to note a gradual decrease in pulmonary compliance during the night as the result of inadequate suctioning of respiratory tract secretions. It may require several hours in the morning to recover the ground lost during the night.

This review will deal with the continuous monitoring of patients in respiratory failure, although it must be admitted at once that the state of the art is primitive. At this moment, for example, there is not available a satisfactory method for the continuous measurement of the most essential parameter in respiratory care—arterial blood oxygen tension. Nonetheless, much has been learned, and recent visits to various laboratories suggest that the future will see gratifying developments in the field, hopefully, soon.

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The review will focus attention on clinical applicability rather than research. It will become evident that equipment for clinical use should be small, stable, rugged, safe, relatively inexpensive, not too complex, and offer few artifacts. Such apparatus is nonexistent. Yet, why should the identification of such life-threatening situations as the disconnection of a respirator from an apneic patient, the dislodgement of a tracheal tube into a main stem bronchus, or the accumulation of secretions leading to major increases in airway resistance require delicate, unreliable, complicated equipment?

It must be emphasized that the monitoring of respiration is much easier in the presence of an orotracheal, nasotracheal, or tracheostomy tube and rather difficult in their absence. Finally, it must be realized that there is a great difference between the "environmental requirements and constraints on patient and instrumentation in an intensive care area, as distinct from a laboratory setting such as the cardiac catheterization laboratory. The patient must be as unencumbered as possible, free to move—indeed, stimulated to move—and easily accessible to the nursing and medical staff." "Electrical drift, temperature instability, changing patient position, sensor contamination and calibration demands constitute problems in an intensive care unit greater by several orders of magnitude than in the catheterization laboratory" (8).

Direct observation represents the oldest technique of continuous monitoring, and there are many who contend persuasively that the human mind is still superior to the machine so far as interpretation of evidence is concerned. For practical purposes, however, there are certain disadvantages in reliance solely on observation. There is, for example, the lack of personnel, the difficulty of an individual's sustaining close attention for long periods, the unreliability of cyanosis in the detection of hypoxia, and the inability to recognize readily moderate degrees of respiratory depression or obstruction.

One turns then to equipment. What can be measured, how useful is the measurement, what are the problems, and what can one hope for in the future?

DETECTION OF THE ABSENCE OF RESPIRATORY "ACTIVITY"

A variety of devices have been used with the hope that cessation of breathing could be detected instantly whenever it occurred. These have included a chest band, balloon (10, 33) or rubber tube containing graphite (45) placed round the chest to record expansion of the chest or lack of it. The devices have been designed to alter strain gauges or electrical resistances and to sound an alarm if apnea developed. One apnea monitor devised for infants took the form of a ripple mattress in which air movement was detected in small air-containing compartments (23). No attachment to the body was required and the little patient could rest in any position. The measurement of impedance across the chest (to be discussed more fully in the next section) has also been used as a continuous monitor of apnea in newborns (44). Of 708 alarms in one study (44) there was a 7 percent incidence of false positives but in no instance did apnea occur without the alarm sounding.

The chief objection to this type of approach is that it does not measure air flow in and out of the lungs. In the presence of complete respiratory tract obstruction any but a paralyzed patient will respond with considerable muscular movement and body motion as he fights against the obstruction. The monitoring devices would continue to record "activity" and a dangerously false sense of security could result.

Thermistor sensors placed in the air-flow tract can be cooled by the exhaled breath. The thermistor's resistance is thereby increased, and the change in voltage can be recorded continuously and fed into a rate meter. An alarm can be made to sound if apnea occurs, or if the respiratory rate decreases below a preset value (3, 17, 27, 45). The sensors may be used in front of the nose, mouth, within the nostrils or attached to an oxygen mask. While properly placed thermistors can detect apnea, or monitor rate changes, even small air currents may activate them and they are unreliable therefore for monitoring reductions in tidal volume as the result either of partial respiratory obstruction or respiratory depression.

RESPIRATORY VOLUMES AND AIR FLOW

Several techniques have been applied to the continuous measurement of tidal volume in the absence of an artificial airway. Impedance spirometry, for example, detects electrical impedance changes occurring across two electrodes placed on opposite sides of the thorax. A constant low intensity sinusoidal current (20–25 kHz) applied to the electrodes insures that the potential change is directly proportional to the impedance change (1). Calibration for measurement of volume can be accomplished with a wedge spirometer or pneumotachograph for spontaneously breathing patients (28) and a large syringe for paralyzed patients (1). Unfortunately, the quantitative correlation between the change in the output signal and the change in the volume of air in the lungs is uncertain in our opinion.

Changes in the anterior-posterior diameter of the rib cage and abdomen are being sensed by a magnetometric system in some institutions. If the two values are summed, it is alleged that an output nearly linear with change in lung volume can be obtained (26). The system in essence measures at one surface the strength of a magnetic field generated at the opposite surface. Since the output of the abdominal magnets is summed with that of the chest magnets, complete respiratory obstruction results in no recorded change, the alterations in the two areas canceling one another. This technique of continuous monitoring seems to merit further study in intensive care units.

Various methods are available for measuring tidal volume continuously with an airway in place. Anemometer recorders, e.g., the Wright meter, measure volumes but readings are affected by moisture and inertia. With this instrument at flow rates of less than 3.8 liters/min or respiratory rates of over 40/min, errors of measurement occur (14). Write-outs and alarms have not been adapted to anemometers. Gas meters have been used continuously with respirators and when combined with heating elements can function continuously without interference from moisture. A gas meter has been adapted to avoid the need to write down dial readings (32). This was accomplished by removing the dial indicator and replacing it with a light interrupter disc. The output is integrated and fed into a chart recorder. A Bellows spirometer used for continuous use with one respirator (Bennett MA-I) has been adapted to a low voltage electronic sensing device, connected to a display warning at the nurses' station. Upper and lower limits of tidal volume and rate can be set with an alarm sounding should either fall outside the preset values. Performance of the instrument has not been as good with infants and small tidal volumes, as with adults (13). Wedge spirometers have also been used to measure respiratory volumes continuously (24, 25).

Any instrument measuring volumes on the expiratory side of a respiratory must consider compliance of the tubing. Currently available disposable tubing gives falsely high values to volume readings especially at high airway pressures.

Respiratory air flow may be measured by a pneumotachograph and integrator. The pneumotachograph is a tube in the lumen of which there is a specially constructed screen through which gases pass easily. This has the effect of imposing a very slight resistance to flow which is physiologically negligible (0.1–0.5 torr) but allows measurements to be made. The aerodynamic characteristics of the screen are such that the pressure across it necessary to maintain the rate of flow of gas is directly proportional to that flow. Pressure is sensed by a transducer and an output signal is obtained whose magnitude is proportional to the instantaneous flow rate. The total volume of gas which passes through the screen during one phase of respiration results from all of the instantaneous rates of flow which have occurred for transient periods of time. Thus, if the output of the transducer is integrated with respect to time throughout that phase tidal volume can be measured (5). The Fleisch instrument is used most frequently with six sizes now available.

The instruments have flow limitations above which linearity is lost. Condensation of water within the instrument can cause turbulence and Fleisch pneumotachographs are therefore heated to minimize condensation. A number of workers have recommended special precautions to improve accuracy. Osborn and colleagues (29) warm the pneumotachograph head to 38°C, reverse flush with warm air between analyses and re-zero the differential strain gauge before each measurement. The differential pressure transducer seems to be the weakest link, with the need being for greater stability and accuracy. Since changes in temperature, humidity, composition of gases and pressure all affect the unit Dammann and co-workers are correct in concluding that the device is difficult to use, and may, for example, be impossible to apply to such goals as a breath-to-breath estimation of oxygen consumption (8). Nevertheless, the pneumotachograph looms large in current practice and has improved continuous monitoring considerably when an artificial airway is in place.

A flow meter with a different principle of operation has recently been introduced into clinical use. This is a mass gas flow meter. The flow of gas is measured by the cooling of a heated thermistor as the gas streams over it. The thermistor, however, is returned to a constant temperature by an increase in electrical current. The change in current is a function of the flow of gas. The current change is converted into a linear signal which is directly proportional to the flow of gas. Accuracy for these flowmeters is alleged to range from 1 to 5 percent (2, 20, 39). The flow is unaffected by varying mixtures of oxygen and nitrogen because the thermal properties and densities of the two gases offset each other whatever their proportion. The transducer is claimed to be impervious to water vapor and ambient temperature and pressure variations. The output of the transducers can be electrically integrated to volume allowing display of tidal or minute volume. Alarms can be adapted to signal low volumes or apnea. The role of such units in continuous monitoring remains to be determined.

MONITORING OF OXYGEN IN THE GAS PHASE

The measurement of oxygen concentration either in the inspired or expired gas is important in the management of respiratory failure. Oxygen should be used as a drug and given in known concentrations, since either too much or too little can be harmful. Paramagnetic or polarographic analyzers have been widely used for the measurement of oxygen concentration in gaseous mixtures, but their application has usually been in an intermittent fashion. Recent developments have allowed the rapid continuous analysis of oxygen concentration in gaseous mixtures (11, 21, 36).

Updike, Dammann & Bowers have studied extensively the performance of a fast response respiratory oxygen sensor manufactured by the Westinghouse Corporation (MDO 3) (43). An earlier model (203C) had been evaluated by Elliott (9). The analyzer measures oxygen in a gas-to-gas concentration cell where chemical potential can be equilibrated against electrical. These instruments have a sufficiently fast response time to allow breath-to-breath analysis of oxygen concentration. The later design is more accurate, has a faster response time and a lower sampling requirement than the earlier model. Updike and colleagues found agreement with a Shollander analysis within 0.2 percent. Response time was 90 percent complete within 50 to 100 mm/sec. A similar oxygen analyzer (zirconium oxide fuel cell) which is small (400 g), can be mounted in any position and is unaffected by vibration has been reported to be equally as accurate and rapid as the Westinghouse Model (38). Zirconium oxide becomes permeable to oxygen ions at high temperature. A fuel cell is made by coating the internal and external surfaces of a tube of zirconium oxide with a porous film of platinum; PO_2 differences on the two surfaces generate an emf in accordance with the Nernst equation $E = RT_{nF} \log P_1/P_2$ where P_1 and P_2 are the oxygen pressures applied to the inner and outer surfaces of the cell. Unfortunately, the cells age fairly rapidly, the high operating temperature (ca 950°C) can be hazardous in the presence of explosive mixtures, or can cause errors due to the consumption of oxygen by combustion (4).

Many rapid response gas analyzers are sensitive to pressure changes. This may cause base line shift if gas is sampled while a patient is receiving positive pressure from a respirator. A flow-regulating valve of small dead space for use in the sampling line has been developed to maintain constant flow over a wide range of airway pressures (31).

Monitoring of Carbon Dioxide in the Gas Phase

Infrared CO_2 analyzers have been used for continuous monitoring by many (6, 16, 18, 30). They offer a short response time (0.1 sec) and can be fairly accurate (ca 0.1 percent CO_2). As Burton (4) points out, however, they are liable to three sources of error:

Overlap phenomenon. Although carbon dioxide has a specific absorption band (at 4.3μ), large adjacent peaks (e.g., nitrous oxide, at 4.5μ) can cause errors due to absorption at common wave lengths. This can be prevented by using the interfering gas as a filter.

Collision (pressure) broadening. Inelastic collisions between molecules of carbon dioxide and of other gases can broaden the absorption peak, causing an error which is proportional to the carbon dioxide concentration.

Nonlinearity. The output from the detector is nonlinear and the calibration of the instrument requires occasional checking. To do this, a wide range of P_{CO_2} values can be obtained using exponential wash-out curves derived from a single standard gas.

The infrared units are, however, quite helpful when properly used. With an infrared analyzer one can measure mixed expired gases continuously or obtain continuous readings of end-expired values. The continuous sampling of mixed expired gas requires mixing devices. One such apparatus consists of polyethylene Buchner funnels joined together with baffles interposed (37). Mixing is supposed to be virtually complete with flows up to 20 liters per minute. Another mixing device consists of a series of 400–500 ml bags, 2 or 3 of which are filled with pieces of nylon (42). Sampling takes place between the two distal bags. The technique compares well to specimen collection in a Douglas bag. Parenthetically, the moxed expired analysis is useful for the measurement of $V_D,/V_T$, $\dot{V}_a$, or $\dot{V}co_2$.

For patients with relatively normal lungs end-expired carbon dioxide measurements have been useful in the adjustment of respirators. By direct recording of end-tidal Pco_2 wide swings in arterial Pco_2 can be avoided.

MONITORING OF PO_2, Pco_2, AND pH OF BLOOD

The increasing intermittent use of polarographic and glass electrodes to measure blood PO_2, Pco_2, and pH has been of inestimable value in the management of patients with respiratory failure. But a reliable method for recording these values continuously is not yet available for use in patients despite the work of such individuals as Henningsen (15), Ledsome (22), and Sugioka (41) who are among those who have tried to withdraw blood from an artery, pass it through an electrode system and return it to a vein. The need to use systemic anticoagulants, and the inability to demonstrate accuracy for more than 6-8 hours has been discouraging. No more success has greeted the introduction of a catheter tip or probe directly into a blood vessel, although again considerable effort has been expended in this direction (34, 35, 46).

Dammann, Updike & Wright (8) are testing disposable chemical sensors, one of which they hope can be used for the continuous measurement of oxygen tension in blood. The sensors are fabricated by techniques developed for semiconductor microcircuits and optical devices. The units are alleged to be small, rapid in response, cheap and accurate but are obviously still in the developmental stage. Dammann's group aims to incorporate such sensors into an external A-V shunt or even better into a catheter tip.

Commercial oximeters with an ear piece as a sensor are available for the continuous measurement of the oxygen saturation of hemoglobin. Disadvantages of this technique include the difficulty of keeping the ear piece in place, problems in calibration and the need for adequate blood flow through the ear. In addition because of the shape of the oxyhemoglobin dissociation curve, changes in saturation are minor over an important portion of the oxygen tension values of interest in respiratory failure.

A bloodless system for monitoring Pco_2 continuously through the skin has been reported. The skin is rendered permeable to carbon dioxide by degreasing with acetone or ether. The "barrier layer" is then stripped off with cellophane. A gas-type, liquid-filled chamber is applied to the skin. Carbon dioxide is sensed in the chamber with a Severinghaus electrode. Approximately eight minutes are required for equilibration of the chamber and the Pco_2. The system is claimed to be useful for continuously monitoring adequacy of ventilation, though no reports of its clinical use have been made (19).

CONTINUOUS MONITORING SYSTEMS

The on-line continuous processing of respiratory data by computer has been the goal of many. With the use of computers raw data can be rapidly processed allowing the continuous display of derived parameters. Data can be stored for later retrieval and retrospective analysis. With proper programming diagnoses can be suggested, alarms activated, and feedback mechanisms utilized.

Several groups of investigators have pioneered in these efforts and a brief description of their systems will be presented. Hilberman, Schill & Peters (16) at Chapel Hill measure continuously lung mechanics, air flow (Fleisch pneumotachograph), and transpulmonary pressure via a differential pressure transducer with one side connected to an esophageal balloon and the other to the airway. Using a digital computer, calculations of airway resistance and compliance are made. In addition, from measurements of expired P_{CO_2}, airway pressure, and expired volume they have developed a control system to adjust respiratory rate, tidal volume, deadspace and cycle-timing for patients on respirators. The physician retains the ability to override the controller. Future plans call for the additional input of blood gas, pulmonary shunt, respiratory mechanics, and cardiac output data to determine adequacy of ventilation and the appropriateness of the controller's response. Most of the clinical application of the system has been to patients following operation. As might be expected, deterioration in pulmonary mechanics in individuals after intra-abdominal procedures correlated well with the development of fever, the production of sputum, and radiographic evidence of atelectasis.

Osborn and colleagues (9, 29, 30), in San Francisco, monitor lung mechanics and gas exchange continuously in patients. They describe two systems, one considerably more complex than the other. In the first, respiratory flow measurements are made with a Fleisch pneumotachograph and gases are sampled through a special regulating valve which maintains a constant flow over a wide range of input pressures (31). The cases are fed into rapid oxygen and CO_2 analyzers. Airway and esophageal pressure are sensed with a pressure strain gauge. The signals from the sensors are recorded by a tape recorder and the tapes played into an analog computer (EAITR 20). From the flow and pressure signals computation of compliance, nonelastic resistance, work of respiration, and tidal and minute volume are made. From the integrated flow signal and values for concentration of O_2 and CO_2, CO_2 excretion, and O_2 consumption are computed.

The more elaborate system includes certain cardiovascular parameters. This seems reasonable since adequate gas exchange in the lung is of little value if tissue and oxygen blood flow decreases below critical values. The circulatory measurements will not be described in this review, however.

Using essentially the same sensing devices for respiratory measurements as are used in the more simple approach, the second system involves the transfer of data to a digital computer (IBM 1800) and computations are made of respiratory rate, minute and tidal volume, work of inspiration, compliance, airway resistance, oxygen uptake, carbon dioxide production, end expiratory CO_2 partial pressure, inspired and alveolar oxygen partial pressure. The information is displayed continuously in both analog and digital form in an intensive care unit as well as in the form of a 24-hour numerical print-out. Alarms are triggered when tidal volume decreases below a set minimum or if airway pressure exceeds a set value to indicate disconnection or obstruction.

In both systems a catheter is placed in the high right atrium usually via the external jugular vein and this position confirmed by X ray. A catheter is also placed in the central aorta, usually by percutaneous insertion via a femoral or radial artery.

The respiratory measurements are believed by this group to have been clinically valuable in the management of respiratory failure. Respiratory adjustments have been made in response to the data obtained and changes in values have initiated clinical searching which has uncovered such complications as hemothorax. The nursing staff has had to become familiar with the apparatus gradually, and the mass of data available is not yet really manageable. The investigators write, "it had been expected that the ability of the computer to display any particular variable at the choice of the physician in any particular form (numeric or graphic) would make his tasks lighter. Instead it has tended to overload the busy and harassed individual by forcing him to try to remember what the normal values of a whole set of new measurements should be. It is planned to 'teach' the computer to call attention to and display important variations from normal and at the same time show what the normal range is." Steps in this direction have been taken by others and "conversations" now take place between physician and computer. The obvious limiting factor in this situation is how much the computer "knows."

Dammann and colleagues (6–8), in Charlottesville, have worked extensively with continuous computer-based monitoring of respiration. At the moment they measure continuously arterial pressure, air flow and airway pressure (patient on Engstrom respirator), airway O_2 and CO_2 concentration, ECG, skin and rectal temperature, and blood gas tensions and pH intermittently. From these variables are computed and constantly displayed pulse rate, systolic and diastolic pressure, lung compliance, airway resistance and the gradient between skin and rectal temperature (as an index of peripheral vasoconstriction). They plan to add oxygen consumption, CO_2 production, blood flow, cardiac work, and blood electrolytes (31). Two major weaknesses in continuous monitoring have been identified. One is the fact that "sensing devices now available are not particularly stable, lack accuracy, are not sufficiently rugged or easy to handle, and are too expensive." The second is the time constraint imposed by computers which makes discrimination against artifacts and signal fluctuations difficult during on-line monitoring. In their stimulating papers are described imaginative approaches to the solution of these two problems. Despite the concerns expressed, however, they contend that the life-saving value of continuous monitoring of respiration has been demonstrated to their satisfaction. Future developments from this group will be watched with a great deal of interest.

HAZARDS OF MONITORING

Hazards to the patient undergoing continuous monitoring have been stressed by many (6, 8, 29, 40). Of particular concern are electrical hazards. Standards of the (*a*) National Electrical Code, (*b*) National Fire Protection Association, and (*c*) National Board of Fire Underwriters do not cover intensive care units (40). Normal safety practices are not adequate when patients have indwelling vascular catheters or implanted electrodes. To eliminate the danger of inducing ventricular fibrillation special precautions must be taken. The use of isolation transformers with a ground current detector alarm is common in operating rooms and has been reported in intensive care units (6, 29). With this precaution if a power lead should come in contact with the frame of the monitor it would merely ground that side of the power line, and sound the alarm. Only a negligible current would flow through the safety ground connection, giving rise to only a few millivolt frame potential with respect to the room ground. Leads can be provided with series resistors and diode limiters which will protect the patient from faulty equipment (29). Low current, fast-blow fuses (5 milliamps) are used in patient cables (6). Systems are designed so that multiple faults must occur before a current path can be established through intravascular catheters (6). When computers are used these can be isolated electrically from the patient (29). A technique developed at the University of Virginia shows promise of being quite safe with intravascular or intracardiac leads (8). The system consists of a preamplifier and modulator attached to the patient, which is battery-operated and can under no circumstances develop more than 5 microamps. The pulse frequency modulated output is a light-emitting diode transmitting through 10 feet of fiberoptic "light wire" to line-powered demodulator and amplifier. Because of the fiberoptic "light wire" the ends are electrically isolated so that line power can be used safely for receiver, recorder, and other distal systems.

Additional threats to the patient being monitored continuously include infection via the vascular catheters and thrombus formation. The use of sterile equipment and sterile procedures for their placement can minimize but probably not eliminate the problem of infection, while proper flushing techniques and the use of smooth, nonwettable surfaces can reduce thrombogenesis (6).

FUTURE DEVELOPMENTS

Use of telemetry, the development of new devices such as ultrasonic flowmeters and of improved chemical sensors, engineering improvements in currently available measuring devices such as the miniaturization of the mass spectrometer, and better handling of data by computers are either awaiting application or are in the wings. While all workers in the field agree that one has merely scratched the surface of continuous monitoring techniques and applications, there is optimism for significant advances in many quarters. Progress will not be made by physicians alone, but by physicians working closely with engineers, data-processing specialists, management researchers, and nurses at the least. Nor should this be the only direction that research in the area of respiratory failure management should take. It is a challenging direction, however.

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METABOLIC MODIFICATION OF PARKINSON'S DISEASE AND OF CHRONIC MANGANESE POISONING[1]

7020

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INTRODUCTION

The reasons for a wedding are not necessarily the same as those that sustain the marriage. The neurochemical leads that preceded clinical experiments with L-dopa in Parkinsonism resemble the former, whereas the impetus for promoting investigations and therapy with this drug resemble the latter.

Early investigations were characterized by a systematic testing of selected hypotheses. These differentiate L-dopa sharply from some other great drugs, which were discovered serendipitously. When L-dopa was brought into the experimental therapy of Parkinsonism, unexpected phenomena such as involuntary movements began to emerge. It has been often impossible thereafter to predict accurately the effects of L-dopa on specific symptoms in different patients. This widespread experience proves that we do not know the mechanisms by which L-dopa acts in man. Since, however, the systematic selection and testing of hypotheses have brought us the drug L-dopa, the same process may serve in capitalizing on the research tool L-dopa. The present review offers us the opportunity to formulate suggestions in that direction.

As compared to such final solutions as poliomyelitis vaccine, L-dopa is a midpoint en route to the conquest of Parkinsonism and of related conditions. As this article indicates, it seems probable that L-dopa will facilitate the advent of a final solution here too. To provoke some action in that di-

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rection, the literature being quoted was selected arbitrarily. An explanatory background is followed by some metabolic, neurochemical, and physiological considerations. The treatment of patients with L-dopa is discussed only to promote its eventual comprehension, improvement, and replacement by even more effective regimens. Selected needs and possibilities are stressed in the conclusion.

BACKGROUND

Biogenic amines (1, 2) and manganese (3, 4) are connected by the following: (*a*) both control related vital processes despite their scant concentrations in tissues (4, 5); (*b*) monoamine oxidase (2) and manganese (3) concentrate in the mitochondria; (*c*) a metabolic link between manganese and biogenic amines is 3′,5′-cyclic AMP (6); (*d*) manganese deficiency in animals (7) and chronic manganese poisoning in animals (8, 9) and in man (10) produce neurological syndromes in which the metabolism of biogenic amines and of manganese is probably connected.

Symptoms encountered in Wilson's or Parkinson's disease are sometimes displayed in chronic manganese poisoning (4, 10), but studies of human manganism presupposed some technical and physiological capabilities. Measurements of this scarce metal in clinical samples have been achieved with neutron-activation analysis which guards against both loss and contamination (11, 12). Some dynamic and homeostatic aspects of the metal's metabolism have been investigated with appropriate radioisotopes (3, 13–19). Studies in man showed that metal-sequestering agents are inapplicable to the neurological phase of manganism. The excesses of metal acquired while mining are cleared upon the miner's removal from the mines (20), whereas the neurological symptoms are not. A metabolic therapy was not formulated initially because of lack of analyses of brains from patients with manganism. In Parkinson's syndrome, however (21), low melanin concentrations in the substantia nigra had been discovered (22, 23). Reserpine had induced Parkinsonism (24) while depleting the tissues of serotonin (25, 26). Carlsson had discovered that dopamine (3,4-dihydroxyphenylethylamine) exists in animal brains (27); that it was diminished by reserpine (28); that this diminution could be reversed by administering the amino acid 3,4-dihydroxyphenylalanine (dopa); and that this reversal corrected the sedation caused by reserpine (28). Ehringer & Hornykiewicz reported thereafter that dopamine and norepinephrine were diminished in the corpus striatum of patients with Parkinsonism (29). In the early 60's (30, 31) intravenous injections of L-dopa were reported to induce short-lived improvement in Parkinsonism, but this was not confirmed by double-blind tests (32, 33). Oral administration was judged ineffective (34, 35) and some critics (36), including some of the original authors (37), appeared discouraged by the therapeutic impotence of dopa.

Attempts to increase the melanin concentration in the brains of these patients with the melanophore-stimulating hormone β-MSH darkened the skin and aggravated the tremor (38). Since several actions of β-MSH are antagonized by melatonin, the latter was administered to a patient with Parkinsonism. It controlled the tremor but caused excessive sedation. In view of the fact that under the influence of β-MSH the skin could have been diverting precursors of melanins from the brain, melatonin was set aside in favor of such precursors. Phenylalanine was ineffective or it, too, aggravated the symptoms (39), suggesting that it was not being sufficiently hydroxylated in the brains of these patients. Tyrosine was skipped, and 3,4-dihydroxyphenylalanine (dopa) was tested thereafter. This had to be converted by brain enzymes (Fig. 1) into active metabolites before it could act. Its dose would therefore depend on undetermined enzymatic activities, among other factors. Saturation of such enzymes was tried by very grad-

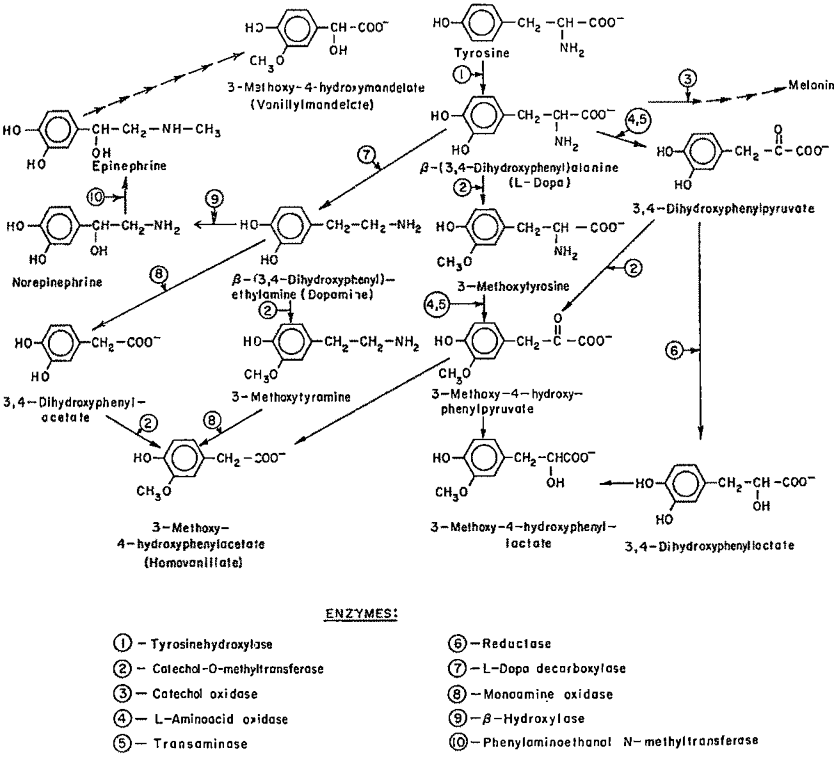
3-Methoxy-4-hydroxymandelate (Vanillylmandelate)
Tyrosine
Melanin
Epinephrine
β-(3,4-Dihydroxyphenyl)alanine (L-Dopa)
3,4-Dihydroxyphenylpyruvate
Norepinephrine
β-(3,4-Dihydroxyphenyl)-ethylamine (Dopamine)
3-Methoxytyrosine
3,4-Dihydroxyphenyl-acetate
3-Methoxytyramine
3-Methoxy-4-hydroxy-phenylpyruvate
3-Methoxy-4-hydroxyphenylacetate (Homovanillate)
3-Methoxy-4-hydroxyphenyl-lactate
3,4-Dihydroxyphenyllactate
ENZYMES:
① - Tyrosinehydroxylase
② - Catechol-O-methyltransferase
③ - Catechol oxidase
④ - L-Aminoacid oxidase
⑤ - Transaminase
⑥ - Reductase
⑦ - L-Dopa decarboxylase
⑧ - Monoamine oxidase
⑨ - β-Hydroxylase
⑩ - Phenylaminoethanol N-methyltransferase

FIGURE 1. Known metabolic pathways of L-dopa. Single arrows denote the action of the enzymes shown by the numbers. Multiple arrows denote multiple enzymes not mentioned in the text. The known metabolic products are named. Note the multiple pathways leading to production of homovanillate.

ually and slowly increasing the daily doses from very small to very large ones. This slowness made the difference between success and failure both in Parkinsonism (38, 40, 41) and in chronic manganese poisoning (42).

GENERAL METABOLIC CONSIDERATIONS

The hydroxylation of L-tyrosine by tyrosine hydroxylase (43–45) is generally the first step in the biosynthesis and metabolism of L-dopa (Fig. 1). This enzyme is primarily bound to synaptic vesicles localized in nerve endings (46) where dopamine and norepinephrine also localize (47), each mainly in its own neurons. The K_m of the hydroxylase in adrenal glands suggests that the enzyme is saturated under normal conditions in that organ, but not necessarily in brain. The lability of this enzyme has precluded its study in autopsy materials so that its role in the pathogenesis of Parkinsonism is unknown. The coenzyme for tyrosine hydroxylase is one of the tetrahydropteridines (48). The purified enzyme requires oxygen, Fe^{++} (43), and reduced pteridine (48), the latter as a coenzyme. Chronic coadministration of these pteridines and of L-tyrosine to Parkinsonian patients may serve to establish whether this enzyme is inactive in Parkinson's disease. The beneficial effect of amphetamines on Parkinsonism is perhaps germane, since they can induce increased activity of this hydroxylase (49). Conversely, inhibition of this enzyme by appropriate inhibitors may have opposing consequences. Inhibition has been shown by means of C^{14} tyrosine conversion to norepinephrine (50, 51) and by the disappearance of histochemically demonstrable brain catecholamines (52). Alpha-methyltyrosine and its methyl ester are potent inhibitors (53, 54), the chronic administration of which in amounts larger than those used elsewhere (55) may be worth testing in animals with the hope of inducing Parkinsonism.

Dopa decarboxylase generates the corresponding amines from dopa and, among other aromatic L-amino acids, from 5-OH tryptophan (56). Despite its consisting of several isoenzymes (57), each with different substrate affinities, it may be subject to substrate competition which may explain the episodic block of the L-dopa effect in some patients following meals. The existence of several isoenzymes may explain why its coenzyme (pyridoxal) activates the decarboxylation of different substrates to different degrees (58) but not why administration of this vitamin to patients can cancel the effects of L-dopa (59). This negative effect is compatible with an increased transamination of L-dopa for which the vitamin is also a cofactor.

Human dopa decarboxylase is also labile in autopsy materials (60, 61). Its activity is, however, readily measurable in fresh animal tissues in which it is almost ubiquitous (56). The vascular decarboxylase has received attention (62). The high vascular concentrations of dopamine induced by L-dopa were reduced with an inhibitor (63) which tends not to enter the brain (64). The further conversion of dopamine into norepinephrine is apparently scant following administration of L-dopa (65) to patients with Parkinsonism, who synthesize less norepinephrine from administered dopamine than do control subjects (66). However, large doses of dopa have induced marked diminution of brain serotonin in animals (67, 68). These latter observations are perhaps relevant to the anti-Parkinson effects induced in one of our patients with the serotonin analog melatonin (69).

Dopamine can readily undergo oxidative deamination (70) but the isoenzyme of monoamine oxidase (71), mainly responsible for this reaction in vivo, is unknown. Inhibition of monoamine oxidase remains a potential option in treatment with L-dopa (72, 73), although monoamine oxidase inhibitors by themselves have not yielded impressive results.

The transmethylation reactions of catecholamines and of their precursors have been extensively reviewed by Axelrod (74) but they will be detailed here because they are deemed central to therapy with L-dopa. The amino acid itself can be extensively O-methylated after administration (75). Blocking of O-methylation of L-dopa has been suggested as a means of potentiating the cerebral effects of the amino acid (76). Judged by the formation of 3-methoxytyramine versus that of normetanephrine, dopamine is even more readily methoxylated than is norepinephrine (77). Judged by the excretion of homovanillic acid (41), about a quarter of the administered L-dopa is recovered in the urine as this methoxylated metabolite alone. The enzyme catechol-O-methyltransferase acts on the amino acid, on its successor amines, and on the corresponding phenolic acids (78). The immediate methyl donor for these reactions is S-adenosyl methionine, which became markedly diminished in the brains of animals receiving L-dopa (79). The most popular "inhibitor' of the O-methyltransferase reaction is a substrate to this reaction, namely pyrogallol (80). Since pyrogallol is poisonous, some nontoxic substrates are proposed below.

Many other transmethylation reactions must become markedly altered in tight compartments of the brain and in peripheral pools during therapy with L-dopa. The chief dietary source of metabolically available methyl groups is the amino acid methionine (81) which, however, competes with L-dopa for transport into the brain, as is noted further on. Methionine becomes a methyl donor only after its conversion by enzymes (82–86) into S-adenosyl methionine. This is the essential cofactor in the main classes of transmethylations, namely, the O-methyl and the N-methyl transferase reactions. In these, the methyl group is correspondingly transferred to a phenolic hydroxyl and to an amino group (78, 83, 86). Since both pathways utilize methionine, high turnovers in either one will deplete the methionine pool upon which the other also depends. The tissue constituents thus affected may range from rapidly metabolized small molecules to macromolecules. For example, the conversion of putrecine to spermidine (87) depends upon S-adenosyl methionine. The function of these polyamines in the accumulation of RNA in mammalian systems has been recently documented (88). The cardinal role of transmethylation reactions involving the purine and pyrimidine bases of both DNA and RNA (particularly of mRNA) has also been recently stressed (89–91). That nucleic acids must be widely connected with L-dopa in nature is illustrated by the high concentrations of both in seeds like the velvet bean.

The in vitro mechanisms by which S-adenosyl methionine acts as the transmethylation cofactor have as their absolute requirement the supply of a divalent metal such as magnesium (83, 84, 92) or manganese (93). These metals have often behaved similarly in vitro but not so in vivo (94). Their role in regulating transmethylations in vivo must be further evaluated because of unpublished pharmacological and metabolic interactions between L-dopa and manganese.

Following the donation of its methyl group, S-adenosyl methionine yields homocysteine, among other products (95). In bacteria, de novo synthesis of methionine from homocysteine requires cofactors structurally related to those activating the synthesis of L-dopa from tyrosine, namely 5-methyl-tetrahydrofolates (96–100). This step often requires vitamin B_{12} (101). In mammals, coadministration of both homocysteine (as homocystine) and choline can also induce this de novo synthesis, but administration of choline alone does not suffice. Choline is converted to betaine prior to the methyl transfer and other substances (102–105) can donate methyl groups also.

This oversimplification shows that the chronic intervention of L-dopa into the transmethylation processes in the tissues of patients with Parkinsonism must have far-reaching consequences. Evidence summarized below concentrates on two relevant ideas.

The first is that the only transmethylated metabolite measured thus far in brains from untreated patients with Parkinsonism is homovanillic acid, which is grossly diminished in this disease. Measurements of other transmethylation metabolites can be conducted on postmortem materials but enzymatic activities disappear rapidly after death. The primate brain is rich in catechol-O-methyltransferase (106) and, therefore, stereotactically operated animals may serve to fill this gap. In Parkinsonism, the diminution of homovanillic acid occurs in tight compartments in which other transmethylation reactions are being abnormally favored, probably long before neurological symptoms become manifest. This may be at the root of the macromolecular abnormalities reported by others (107) for the brain in Parkinsonism.

The second idea is that L-dopa is a methyl group acceptor which restores some degree of normalcy to transmethylation reactions and therefore probably to the macromolecular composition of the Parkinsonian brain. This hypothesis is compatible with the following: (*a*) stopping L-dopa does not necessarily result in immediate loss of its therapeutic effects (38, 40, 41, 108); (*b*) continuing the drug seems to slow the otherwise inexorable deterioration of these patients.

In addition to L-dopa, agents which may serve as clinical investigative tools include competing substrates and inhibitors of transmethylation. By the former we mean agents that accept methyl groups and thus deplete the methionine pool. Nontoxic, such agents are D-dopa, the doubly hydroxylated metabolites of L-dopa (Fig. 1), nicotinamide (83), and histidine (109). These may serve to show whether methylation of L-dopa and formation of dopamine are not inevitably coupled.

The O-transmethylation traffic can be specifically impeded with inhibitors which are not themselves methyl acceptors, such as the substituted tropolones. These seven-carbon (instead of six-carbon) ring compounds are almost isosteric with catechol (110, 111), and their inhibitory effects seem to be coupled with their metal-binding properties.

Some lesser pathways besides these major ones may be relevant during therapy with L-dopa. Transamination of L-dopa and oxidative deamination (Fig. 1) have been considered by others (65). The former needs pyridoxal (vitamin B_6) as its necessary cofactor. A minor pathway which may produce powerful metabolites is auto-oxidation (112). This may generate 6-hydroxydopamine, which has caused chemical sympathectomy (113). During treatment, this reaction has probably not been proceeding because, instead of permanent signs of sympathectomy, transitory signs of sympathetic stimulation have sometimes accompanied L-dopa administration. Condensation of dopamine with the aldehyde produced by its own deamination may well induce the synthesis of tetrahydropapaverolinium compounds (114) which seem capable of neurotransmission. These have structural similarities with apomorphine, some effects of which are alluded to below.

NEUROPHARMACOLOGICAL CONSIDERATIONS

The review of some neuropharmacological facts pertinent to L-dopa necessitates brief departures into the gross and microscopic structure of its target organs and into functional processes indigenous to them.

The known connections among the various striatal structures and between them and other parts of the brain have been detailed in treatises by recognized experts (115–117). The afferent neuronal connections include those between (*a*) the cortex and the striatum, (*b*) certain thalamic nuclei and the striatum, and (*c*) the pars compacta of the substantia nigra and the striatum. We are not aware of the nature of the neurotransmitters in the corticostriatal tracts but the others include adrenergic, serotonergic, and dopaminergic neurons. It must be repeated that administration of L-dopa has diminished the cerebral serotonin concentration in normal experimental animals (67, 68). This suggests that chronic therapy with L-dopa may possibly convert serotonergic neurons into dopaminergic ones. This wild speculation may be entertained until a better one is found to explain the emergence of the involuntary movements as a function of the cumulative dose of L-dopa.

Efferent striatal and pallidal fibers as well as striatonigral projections have been demonstrated in a variety of experimental animals (117–121). The integrity of these fibers as well as that of the afferent fiber system appears necessary to the normal control of coordinated movement (122). Both electrical (123, 124) and chemical (125) stimulation of the caudate nucleus have evoked electrical responses in the globus pallidus and probably also in the substantia nigra. These responses have been suppressed by applying L-dopa or dopamine directly to the caudate nucleus (124, 125). Suppression of the caudate's excitability may not offer a complete explanation for the suppression of the symptoms of Parkinsonism by L-dopa, because conversion of L-dopa into dopamine takes place also in other basal ganglia (126) which can also suppress these symptoms.

The dopaminergic nigrostriatal fibers originate in the cell bodies located in the pars compacta of the substantia nigra, known terminals of which extend into the caudate nucleus, the putamen, the nucleus accumbens, and the tuberculum olfactorium (127). A large number of dopaminergic nerve endings are also present in the external layer of the median eminence (128). Whereas the nigrostriatal neurons may be among the target organs in the control of Parksinsonism, those in the median eminence are most probably instrumental in the changes of blood pressure which often accompany such control.

The amine-containing granules, to be detailed below, progress normally from cell bodies down the nerve axons to the varicosities of the nerve terminals (129–132). This has been demonstrated in adrenergic nerves by means of ligation or severance. The granules accumulated proximally to these lesions, and release of the ligature re-established their distal progression. A similar sequence has been demonstrated in dopaminergic fibers of the mammalian brain. Lesions in the substantia nigra (134) or in the tegmentum (133, 135) had the following consequences: (*a*) dopamine tended to diminish distally to the lesion; (*b*) the animals showed contralateral tremor, rigidity, and akinesia; (*c*) administration of L-dopa increased the dopamine concentration only on the normal, unoperated side (136). This does not necessarily disqualify the nigrostriatal terminals as target organs of L-dopa, because, on the side of the lesion, not only the neurons of the substantia nigra disappeared but also some other ones (136).

The intraneuronal distribution of biogenic amines has been subdivided into pools of different functional significance by means of elegant pharmacological methods which have been detailed by some of their originators (137). The most widely studied storage sites for these amines are the granular vesicles. These contain dopamine, norepinephrine or serotonin, depending upon which neurotransmitter is indigenous to which neuron. The granules concentrate largely in characteristic varicosities close to the terminal segments of the nerve fibers (138). A single varicosity contains roughly 3×10^{-10} g of norepinephrine (139) and this concentration falls off proximally where there are fewer granules. Similar distribution and appearance have been demonstrated also for dopamine-containing granules in dopaminergic neurons of mammalian nigrostriatal tracts (140, 141). They, too, seem to be assembled in the vicinity of the nucleus, from where they progress toward the terminal synapses. The average rate of this transit approximates a few millimeters per hour (130–132) and the normal life expectancy of a granule averages several weeks (142).

The release of dopamine by individual cerebral neuronal impulses is extrapolated from experiments with isolated spinal cords (143, 144), brain slices (145, 146), and peripheral nerves (147, 148). All of them suggest that cerebral nerve impulses must indeed release amines from these granules. The estimated amount of amine released per nerve impulse, however, seems to represent an almost negligible percent of the amount calculated for a single granule. This incredible economy, considered together with the large extra-granular pools of "inactive" dopamine, makes it hard to believe that the necessarily large doses of L-dopa restitute only neurotransmission. This doubt is strengthened by the following:

Large doses of reserpine deplete the granules of dopamine. Careful titration with this drug, short of depleting these granules, has severely diminished the total catecholamine concentration in the brain, without inducing behavioral changes or sedation (149, 150). This suggests the existence of large extra-granular pools of dopamine whose function is not neurotransmission. Nonetheless, adrenergic (and by analogy, dopaminergic) neurotransmission is restored following reserpinization only after the uptake of monoamines by the granules is restored (151, 152).

Observations of the transport of the monoamines into neurons of the intact brain are hampered by their susceptibility to enzyme action (Fig. 1) and by the blood-brain barrier. Various indirect methods had to be devised (153–156) which revealed the existence of some selective mechanisms provided by the neuronal membrane for the entrance of dopamine, norepinephrine, and serotonin.

The mechanisms by which neurotransmitter substances (including dopamine) may serve in the normal communication between neurons have been subjects of several recent treatises (157–159). Rasmussen (160) convincingly correlates the features which neurotransmission shares with other types of cell communication, from monocellular colonies to the mammalian endocrine system. Since all these features are involved in the communication between so many kinds of cells, the processes selected or discovered by Rasmussen must be essential to the communication among brain cells. Even the most basic elements of the mechanism which permits communication include all of the following: (*a*) divalent metals like calcium; (*b*) the intracellular messenger, 3′,5′-cyclic AMP; (*c*) intracellular microtubules, microfilaments, and neurotransmitter-containing vesicles; (*d*) a class of enzymes known as protein kinases which phosphorylate specific proteins using ATP as their substrate. Rasmussen clearly shows, therefore, that neurotransmission depends upon the integrity of many biochemical and structural elements, all of which cannot be reconstituted in disease merely by delivering an amine to a receptor.

The striatal concentration of dopamine is higher than that of norepinephrine, whereas the reverse holds true for hypothalamic loci (5, 137). The distribution of homovanillic acid roughly parallels that of dopamine but this diffusible end product is also present elsewhere in the brain (161). Correlations between the localization of some of these substances and of some of the enzymes which produce them have been made (162), but studies of transmethylation in brain remain, indeed, a fertile field. Likewise, in the brains from patients with Parkinsonism, both biogenic amines and relevant enzymatic activities have been measured (29, 163–165). Brains from animals with stereotactic lesions have also been similarly analyzed (133, 135, 166, 167). In the Parkinsonian brain, dopamine, norepinephrine, and serotonin have been reported variably as diminished in the substantia nigra, the caudate nucleus, the putamen, and perhaps the globus pallidus (168). In these and other loci, the depletion of homovanillic acid was very pronounced. The reported normalcy of dopa decarboxylase activity in the brains of patients with Parkinsonism (169) must be re-evaluated because of the lability of this enzyme and of tyrosine hydroxylase after death. The animal experiments alluded to above are of the greatest importance not only because they have brilliantly elucidated normal functions of the brain but also because they have offered superb laboratory models (133, 134, 170). In these and the other animal models referred to here, the physiological changes imposed by one lesion upon those of another lesion and some of the biosynthetic consequences thereof, have been correlated. Depletion of biogenic amines, of tyrosine hydroxylase, and dopa decarboxylase, as well as administration of precursors of neurotransmitter substances, have been most fruitfully investigated.

The uptake of L-dopa by the intact mammalian or human brain has not yet been studied extensively but it may be similar to other large neutral amino acids (171). This assumption is supported by the fact that the uptake of radioactive DL-dopa was reduced in rat brain by competition with L-methionine (172), while similar effects were induced with L-tryptophan. The inhibitory effects of one amino acid on another (173) were reduced by esterifying their carboxyl groups (171), which may explain why the orally given methyl ester of L-dopa enriched animal brains more rapidly with dopamine than did L-dopa (174). N-methylation of large neutral amino acids has changed their transport mechanism to that of small neutral ones (175). This may be useful particularly if N-methyl dopa can be slowly decarboxylated in vivo despite conclusive in vitro evidence to the contrary (176).

Brain has shown higher uptakes of amino acids than other tissues in vitro (177), and their own transport may also be inhibited by L-dopa (178).

The effect of amino acids (as opposed to amines) on cerebral excitability (179) is important in the present context, particularly since their efflux from the brain during the prevalence of high plasma levels has been well documented (180). The regional heterogeneity of the uptake of amino acids such as phenylalanine by brain (181) may also be germane because phenylalanine inhibits the enzymatic synthesis of L-dopa (182).

Inhibition of effects of L-dopa in intact animals can be induced either by impeding its transport or by inhibiting its metabolism within the brain. Conversely, potentiation of these effects can be accomplished by the reverse.

Effects of graded doses of L-dopa have been inhibited in mice during unpublished experiments with oral phenylalanine, tyrosine, or 5-OH tryptophan given in graded doses. All suppressed the motor hyperactivity caused by L-dopa but prevention of mortality diminished in the order of their presentation. Indeed, 5-OH tryptophan actually increased the mortality. Melatonin (0.4 mg/g mouse) eliminated the motor hyperactivity and markedly reduced the mortality from otherwise lethal doses of L-dopa (in unpublished experiments).

Monoamine oxidase (MAO) inhibitors may potentiate the effects of L-dopa in animals and man (172, 183, 184) but inhibition of peripheral MAO is undesirable (73) because dopamine can induce cardiovascular changes (185). Amino acid analogs of MAO inhibitors may perhaps "home" into the brain after blocking their peripheral decarboxylation, as was done already with L-dopa.

Decarboxylase inhibitors have been investigated in animals and in man. N-(D,L-seryl)-N'-(2,3,4-trihydroxybenzyl) hydrazine or RO 4–4602 has acted preferentially on extracerebrally located decarboxylases. It has increased the content of tritiated dopa in the blood and the supply of this amino acid to the brain (186). This is probably the mechanism for the marked accumulation of catecholamines (especially dopamine) in the brain as opposed to the periphery (186). These experiments were extended by the synchronous analyses of the concentrations of C^{14} dopa, 3–0–methyldopa, homovanillic and 3,4–dihydroxyphenyl acetic acids in brain versus peripheral tissues from animals receiving trititiated dopa with and without RO 4–4602 (64). A comparison of this inhibitor with some others (187) has shown that RO 4–4602 remains outside the brain only when given in sufficiently low doses. Among the others, α-methyldopa (Aldomet®) has inhibited only the peripherally located decarboxylases despite penetrating into the brain (187). Its analog, α-methyldopa hydrazine (MK-485), has remained outside the brain over the entire range of concentrations tested (187). MK-485 has potentiated several expressions of the action of L-dopa on the central nervous system, whereas it has inhibited peripheral effects of the amino acid (188). Parallel in vitro and in vivo experiments with MK-485 (189) have shown it to diminish peripheral degradation of both L-dopa

(67) and of 5-OH tryptophan while it, itself, became decarboxylated. Its effects on norepinephrine biosynthesis (190) and on the metabolism of α-methyldopa (191) have been well documented.

CLINICAL CONSIDERATIONS

The medical therapy prevailing prior to the advent of L-dopa has been reviewed by experts (192–194). The first demonstrations of the clinical effectiveness of this drug (38, 40, 41) have been generally confirmed and greatly refined by others (195–214). The objectives of investigators, however, were changed by the release of this drug for general use. It now behooves us to strive for understanding of present therapy and for its replacement by even more effective regimens. The sum of the literature quoted above is, therefore, discussed with that in mind.

In some series, patients were disqualified during the early stages of therapy. Apparently it was not generally appreciated that L-dopa induces progressive tolerance in peripheral tissues, whereas some of its effects on the brain seem to increase with time (215). Examples are nausea, vomiting, faintness, and cardiac arrhythmia, which can be easily avoided by taking it easy with this drug. A generally applicable schedule was therefore presented (216).

Striking mental improvement has been induced early in treatment. By contrast, occasional patients on high doses in most series have shown reversible but adverse mental reactions. In each series, all neurological symptoms have been improved albeit not yet to the same degree in each patient. Whenever ascertainable, the sequence with which major symptoms responded was this: akinesia, rigidity and, after much time and L-dopa, tremor. Ancillary symptoms from seborrhea to dysuria have also been controlled.

The maintenance dose of L-dopa had to be readjusted with each patient's changing needs. A tendency towards lower levels of this dose was at first dictated by the emergence of involuntary movements. Yet this was found also practicable in some of the patients who had been consuming dopa for many years without developing dyskinesia (215). Since their original observation (38), these movements became recognized by all as part and parcel of effective therapy with L-dopa in a significant number of patients with Parkinsonism (217, 218). Despite their emerging as a function of time (or of cumulatively consumed L-dopa, 215), these drug-dependent and reversible movements imitate several of the permanent dyskinesias. Presently, the best method for reducing these movements is reduction of L-dopa. This may, however, reduce also the control of Parkinsonism. Pyridoxine (59, 219) and the other agents listed in Table 1 were either ineffective or they cancelled the beneficial as well as the undesirable actions of L-dopa. Tranquilizers have apparently also followed this pattern. A conservative estimate may be that one-fourth of the Parkinsonians consuming effective doses of L-dopa for

TABLE 1

Drug	Number of patients	Maximum dose per day (g)	Maximum administration (days)	Range of daily dose of L-dopa (g)
Melatonin	1	1.35	51	0
L-Tyrosine	1	12.0	17	3.15–3.95
L-Phenylalanine	2	15.0	4	3.0–8.0
L-Tryptophan	5	7.4	33	3.6–5.2
D,L-5-OH-Tryptophan	9	8.0	114	3.3–6.2
DL-Methionine	4	15.0	11	3.6–7.2
Choline	4	5.85	44	2.8–3.9
Pyridoxine·HCl	12	0.3	124	3.6–6.6

Agents administered to patients with Parkinsonism in more doses than a single oral one. Melatonin controlled the tremor but induced sleepiness. Among the other agents only DL-methionine and pyridoxine had unequivocal effects. These were in the direction of diminishing both the improvement and the involuntary movements (when present) during therapy with L-dopa.

several years have not developed these movements. Furthermore, as of the present writing, the patients with chronic manganese poisoning have not shown even a single incident of involuntary movement (42). The doses of L-dopa, however, the amount of time on the drug, and the clinical improvement have matched or surpassed those of patients with Parkinsonism who have shown dyskinesia. For this reason and for others mentioned below, L-dopa has yielded even greater net benefit to patients with chronic manganese poisoning than to patients with Parkinson's disease. These comparative metabolic and clinical studies of these two kinds of patients are being continued with the hope that they may lead to other complete dissociations between therapeutic effects and side-effects of L-dopa.

The progressive emergence of involuntary movements on steady doses of L-dopa may be viewed together with the following: (*a*) the surprising absence of a sustained "fall-off" phenomenon in the actions of this drug on the brain; (*b*) the striking, continuing increase with passing months of the percent of administered L-dopa excreted as dopamine in the urine (215); (*c*) the persistence of the beneficial effect of L-dopa after its withdrawal in some patients (38, 40, 41, 108). These observations argue that L-dopa leaves a chemical memory of its passage on the tissues, the consequences of which are in the direction of increasing its effects on the brain. This constitutes a sharp difference between L-dopa and other anti-Parkinson drugs, including the nontoxic, useful amantadine (220). An explanation of this difference, presented elsewhere (221), draws upon the fact that L-dopa is a catechol derivative, in contrast to the other drugs. The section on transmethylation of the present review indicates some leads for further studies of this matter on the molecular level. Suffice it to repeat (41) that with an average dose of L-dopa the homovanillic acid excreted accounts for a large fraction of the methionine supplied by a regular American diet (estimated from 222). This was noted earlier (41), but the concern was that chronic administration of L-dopa may cause liver damage by inducing essentially a methionine deficiency. This suspicion was based not only on many animal experiments by others, but also on the fact that in the childhood disease kwashiorkor (223), the liver damage has been primarily ascribed to methionine deficiency. We were unable to show such damage among adults who had been started on DL-dopa five years ago and were kept on the L-compound thereafter. This even raised the question whether L-dopa may not be regulating some pre-existing metabolic abnormality in these patients involving methionine.

The responses to L-dopa in the patients with chronic manganese poisoning seem to have differed from those with Parkinsonism also with regard to blood pressure changes. The former have not yet shown notable hypotension, in contrast to the latter, in whom hypotension of varying severity has been reported both specifically (224, 225) and as a part of more general reports. Like the involuntary movements, hypotension has rarely been severe enough to require discontinuation of the drug. Some hitherto unpublished correlations have been made by one of us (S.D.) between blood pressure and involuntary movements as well as between blood pressure and control of Parkinsonism. These suggest strongly that the hypotensive action of L-dopa is brought about primarily by cerebral mechanisms. That is why the median eminence was mentioned above.

These self-potentiating effects of L-dopa may be kept in mind while considering the following passage, which deals with peripherally acting metabolic inhibitors. Such inhibitors were discussed already with regard to their potentiating effects on L-dopa administered to animals. Two of these inhibitors have shown parallel effects in man (41, 191, 226–228). Their capacity to potentiate L-dopa was demonstrated by their decreasing the effective dose of the drug by as much as 80 percent in certain patients. Reduction of the dose of one drug by another hardly constitutes a medical advance per se. Judging from the literature quoted, but primarily from unpublished personal experience with MK-485, we may perhaps expect the following advances: (*a*) Patients who are refractory to the full effects of L-dopa may become less so with the coadministration of the inhibitor; (*b*) those whose diurnal performance on L-dopa vacillates between extremes may acquire a steadier performance with a combination of L-dopa and MK-485; (*c*) some unwanted effects of L-dopa on extracerebral tissues may become diminished with this inhibitor; (*d*) patients with pre-existing cardiac arrhythmias may perhaps become easier to manage on a combination than on L-dopa alone. Our study is also raising the following reservations: whether MK-485 cannot increase the emergence of both involuntary movements and of hypotension under certain conditions; and whether MK-485 or its metabolites inhibit the decarboxylation of L-dopa specifically as opposed to interfering with transmethylation as well.

A great many references were used thus far in order to challenge the notion that L-dopa improves patients merely by replenishing a missing messenger, namely dopamine. This challenge is pursued further by considering some evidence pertaining to the metabolic fate of L-dopa administered to man (229–232). It appears that several tissue pools, including the cerebrospinal fluid, can become enriched with metabolites of L-dopa long before the drug has exercised its full therapeutic effects. In many instances, furthermore, the levels that these metabolites have reached are much higher than those considered to be normal. Neither one of these considerations seems to conform to precedents from the treatment of hormonal or even nutritional deficiencies.

Although the synthesis of dopamine from L-dopa is not sufficient as an explanation of the drug's therapeutic conduct, this synthesis appears necessary as judged by the circumstantial evidence obtained with apomorphine. Apomorphine is structurally similar to dopamine and is an activator of dopaminergic neurons in the mammalian striatum (233–235). Prior to these discoveries, Schwab et al had shown that injection of this agent can improve the symptoms of Parkinsonism temporarily (236). This was confirmed by us (237), and the following hitherto unpublished results were added since: (*a*) injections of 1–2 mg of apomorphine were tolerated better by patients receiving L-dopa (with and without MK-485) than by untreated ones; (*b*) tremor was the first symptom to be eliminated in all tests, whether or not it was present in untreated patients or in those receiving L-dopa; (*c*) rigidity and akinesia responded later and not always; (*d*) akinetic episodes were readily reversed by apomorphine in patients receiving full dosages of L-dopa; (*e*) involuntary movements were evoked only in one patient who was off L-dopa (237), but they seem thus far diminished in those actually receiving L-dopa.

In addition to the relevance of these experiments to the understanding of therapy with L-dopa, the following practical point has emerged: since tremor was immediately abolished by apomorphine and much later by L-dopa, apomorphine analogs become worthy of synthesis and trial.

CONCLUSIONS

The clinical investigator is being frustrated by his own inability to predict the response of a patient to L-dopa with the accuracy to which he is accustomed when administering antibiotics, digitalis, or insulin. The difference lies in that the mechanism of action of these other great drugs is well known by comparison to L-dopa.

The main reason for this continuing uncertainty is perhaps that the following has not been fully recognized: L-dopa has two different sets of actions, those of a nutrient and those of a drug.

In its role as a nutrient, L-dopa offers particularly fruitful opportunities because it can compete for transmethylations with tissue constituents ranging from small compounds to macromolecules, as was amply stressed.

Elucidation of the role of L-dopa the drug can be materially promoted by further experimentation with apomorphine, which is clearly a drug and not a nutrient. Our urgency to synthesize selected apomorphine analogs is due chiefly to the following: apomorphine injections induce almost immediate disappearance of tremor, namely, one symptom which L-dopa controls last as well as least.

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PAPILLARY MUSCLE DYSFUNCTION IN CORONARY (ISCHEMIC) HEART DISEASE

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Mitral value incompetence due to acquired or congenital disease of the mitral valve leaflets or to interruption of the chordae tendineae, has long been recognized, whereas valve incompetence secondary to disease of the papillary muscles has until recently received little recognition. This review offers a brief discussion of papillary muscle dysfunction based upon studies from the Department of Medicine of the Tulane Medical School during the past 20 years (1–7).

Normal Mitral Valve Function

The mitral valve apparatus consists of (*a*) the mitral valve annulus, (*b*) the valve leaflets, and (*c*) the chordae tendineae and papillary muscles.

The mitral annulus is a dense fibroelastic structure, incomplete anteriorly, ranging in circumference from 18 to 177 mm (8). There are two mitral valve leaflets, a large anteromedial leaflet and a small posterolateral leaflet. The mitral leaflets are not anatomically separate structures, being continuous at the anterior and posterior commissures. The chordae tendineae arise from the apices of the papillary muscles and fan out to insert into the corresponding halves of both mitral valve leaflets.

There are two groups of papillary muscles in the left ventricle. The anterolateral papillary muscle arises anteriorly from the free wall of the left ventricle and receives its major blood supply from the circumflex branch of the left coronary artery and a secondary blood supply from the anterior descending branch (Fig. 1). The posteromedial papillary muscle arises near the junction of the posterior wall of the left ventricle and the interventricular septum. The blood supply to the posteromedial papillary muscle is variable (9). In general, when the left coronary artery is dominant, the posteromedial papillary muscle receives its major blood supply from the circumflex branch of the left coronary artery, whereas when the right coronary artery is dominant, the major blood supply is from the posterior descending branch of the right coronary artery (Fig. 1).

At the end of ventricular diastole the mitral valve leaflets, which were retracted into the left ventricular cavity early in diastole, begin to move

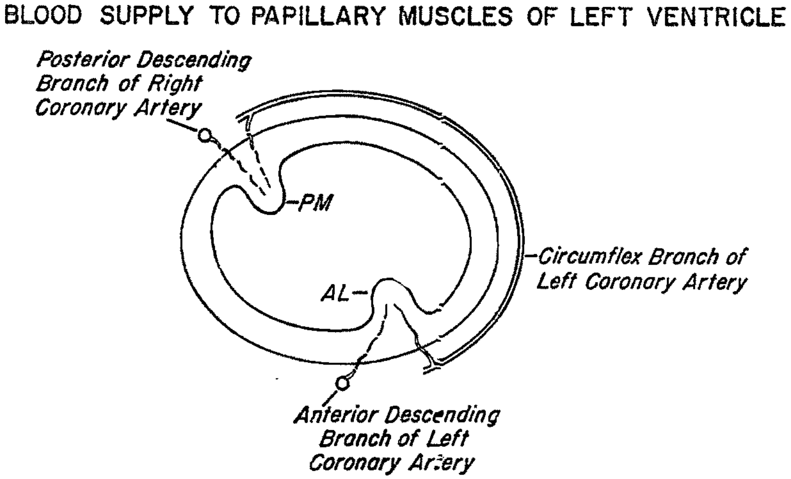

BLOOD SUPPLY TO PAPILLARY MUSCLES OF LEFT VENTRICLE
Posterior Descending Branch of Right Coronary Artery
PM
AL
Circumflex Branch of Left Coronary Artery
Anterior Descending Branch of Left Coronary Artery

FIGURE 1. Diagrammatic representation of the blood supply to the papillary muscles of the left ventricle. (From Burch, DePasquale & Phillips. 1968. *Am. Heart J.* 75:399)

upward toward the left atrium to come into apposition first at their margins and then over a progressively greater area of their atrial surfaces (Fig. 2). Thus, the atrioventricular orifice may be essentially sealed before the onset of ventricular systole (10). With the onset of isovolumetric contraction two opposing forces act upon the mitral valve leaflets, i.e., the centrifugal force of intraventricular pressure and the centripetal force of papillary muscle tension. The net result of these forces is to bring the mitral valve leaflets into firm apposition (Fig. 2).

During isovolumetric contraction the distance between the base of each papillary muscle and the mitral valve orifice changes relatively little. However, during the ejection phase of ventricular systole the apex of the left ventricle moves toward the atrioventricular orifice so that the distance between the base of each papillary muscle and the atrioventricular orifice decreases (Fig. 3). Since the length of the chordae tendineae is fixed, the papillary muscles must shorten to take up the slack which otherwise would have been created in the chordae tendineae during systolic ejection. In other words, because of contraction of the papillary muscles, the distance between the apex of each papillary muscle and the atrioventricular orifice remains essentially unchanged in spite of the fact that the distance between the base of the papillary muscle and the atrioventricular orifice decreases (Fig. 3).

ABNORMAL MITRAL VALVE FUNCTION CAUSED BY PAPILLARY MUSCLE DYSFUNCTION

The function of the papillary muscles to restrain the mitral valve leaflets

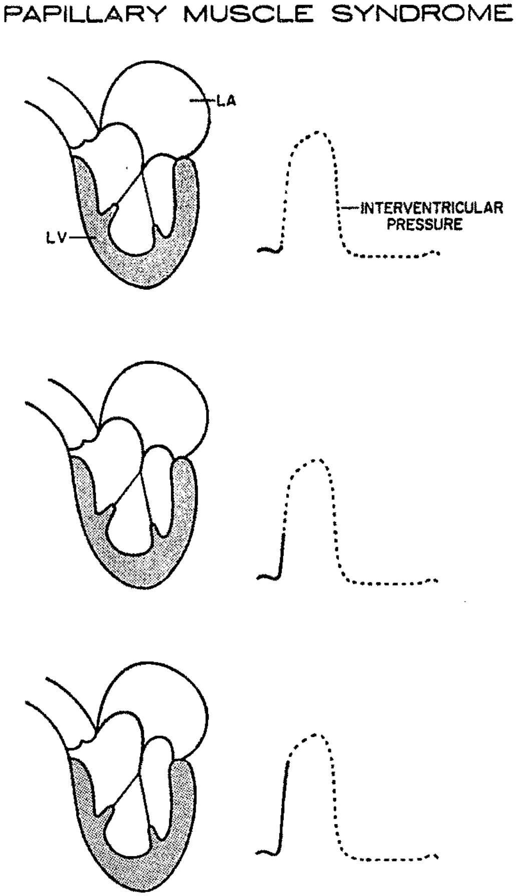


FIGURE 2. Relationship between left ventricular pressure and the apposition of the mitral valve leaflets during isovolumetric contraction. As intraventricular pressure increases, the mitral valve leaflets come into apposition over a progressively greater area of their atrial surfaces. (From Burch, DePasquale & Phillips. 1968. *Heart Bull.* 17:4)

is obvious. However, the dynamic nature of this function has not always been appreciated. A variety of disease processes may alter the spatial relationships between the various elements of the mitral valve apparatus to produce mitral incompetence (Table 1). Because space does not permit a comprehensive discussion of all of the causes of papillary muscle dysfunction indicated in Table 1, only mitral incompetence due to ischemia or infarction of a papillary muscle will be described. With imagination and a knowledge of normal papillary muscle function, the reader can easily understand how

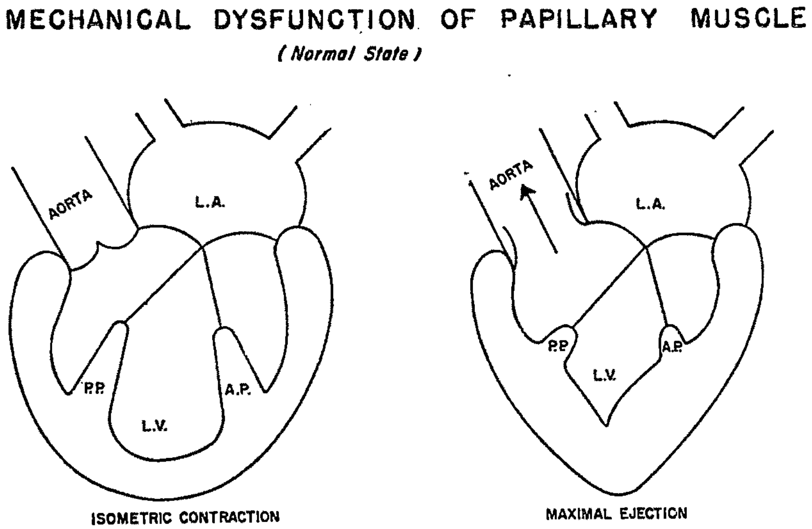


FIGURE 3. Diagrammatic representation of normal papillary muscle function. Although anatomically incorrect, for purposes of simplification, a single chorda tendinea is depicted as inserting into a single mitral leaflet. Actually, chordae tendineae from each papillary muscle fan out to insert into the corresponding halves of both mitral leaflets. During isovolumetric contraction, the mitral valve leaflets come into contact and bulge toward the atrium, pulling the chordae tendineae taut. As the ventricular musculature contracts and shortens during the ejection phase of ventricular systole, the distance between the base of each papillary muscle and the atrioventricular orifice decreases. Because of shortening of the papillary muscles, the distance between the apex of each papillary muscle and the A-V orifice remains essentially unchanged and the chordae tendineae remain taut. (From Burch, DePasquale & Phillips. 1963. *Arch. Intern. Med.* 112:112)

other diseased states may cause papillary muscle dysfunction and mitral valve incompetence.

As already indicated, during the isovolumetric phase of ventricular systole, the mitral valve leaflets come into firm apposition and the atrioventricular orifice is firmly sealed. Infarction or ischemia of a papillary muscle may render it partially or totally unable to contract and shorten. Thus, during the ejection phase of ventricular systole the slack created in the chordae tendineae by the apex-to-base movement of the left ventricle is not taken up by shortening of the papillary muscles. This failure to maintain the chordae tendineae taut allows a portion of each mitral leaflet to evert into the left atrium during ventricular ejection and the mitral valve becomes incompetent (Fig. 4).

The papillary muscles, being subendocardial structures, receive a terminal coronary arterial circulation and are therefore particularly vulnerable to

TABLE 1. Etiology of Papillary Muscle Dysfunction

- Circulatory insufficiency (ischemia)
  - Angina pectoris
  - Infarction of papillary muscle
    - Acute
    - Chronic (fibrosis)
  - Systemic circulatory disturbances (hypotension, erythrocytosis, anoxia, hematometakinesia, etc.)

- Left ventricular dilatation
  - Generalized
  - Localized

- Nonischemic atrophy of papillary muscle
  - Senile
  - Associated with cachexia

- Defective development of papillary muscle apparatus
  - Congenitally long or short papillary muscle or chordae tendineae
  - Ectopic origin of papillary muscle
  - Ectopic insertion of chordae tendineae

- Endocardial disease
  - Endocarditis
  - Endocardial fibroelastosis
  - Endomyocardial fibrosis

- Heart muscle disease
  - Inflammatory (myocarditis)
  - Degenerative cardiomyopathy
  - Infiltrative (metastatic carcinoma, amyloidosis)
  - Neoplastic (primary tumor of myocardium)

- Disturbances in the time course of papillary muscle activation and contraction

- Rupture of papillary muscle or chordae tendineae

ischemia. Furthermore, on the basis of theoretic considerations, it has been calculated that each papillary muscle of the left ventricle supports a total accumulated peak load of about 19 tons during a 24-hour period for a heart rate of 70 beats per minute and an arterial pressure of 120/80 mm Hg (4). It should also be pointed out that during ventricular ejection tension decreases in the free wall of the left ventricle while papillary muscle tension must be maintained and even increased (4). Thus, while the free wall of the left ventricle "loafs" during ventricular ejection, tension must be maintained in the papillary muscles. For these reasons the oxygen requirement

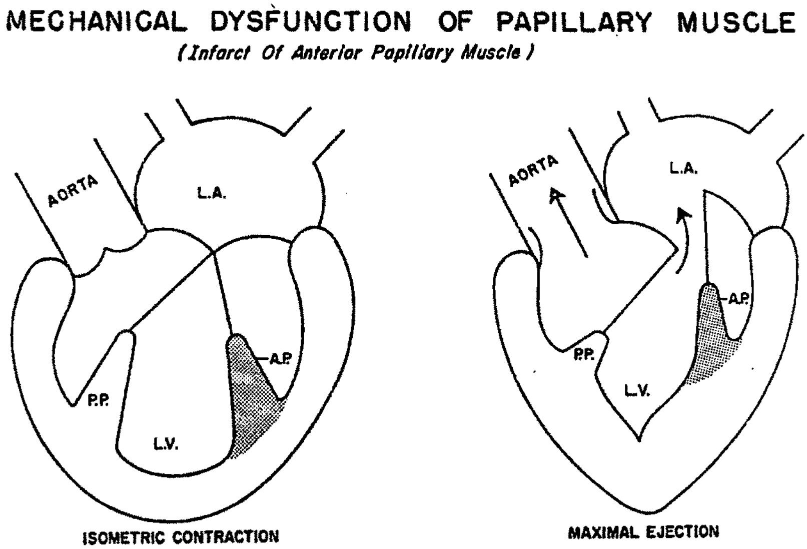

MECHANICAL DYSFUNCTION OF PAPILLARY MUSCLE
(Infarct Of Anterior Papillary Muscle)
AORTA
L.A.
P.P.
L.V.
A.P.
ISOMETRIC CONTRACTION
AORTA
L.A.
A.P.
P.P.
L.V.
MAXIMAL EJECTION

FIGURE 4. Diagrammatic representation of papillary muscle dysfunction due to ischemia or infarction of a papillary muscle. With infarction (or ischemia) of a papillary muscle, the atrioventricular orifice may remain sealed during isovolumetric contraction. However, because the infarcted papillary muscle is unable to shorten during ventricular ejection, the distance between the apex of the infarcted papillary muscle and the mitral valve orifice decreases and a portion of each mitral leaflet everts into the left atrium, causing mitral valve incompetence. Although the spatial relationships between the various elements of the mitral valve apparatus are maintained during isovolumetric contraction, the mitral valve may become incompetent during this phase of systole because of failure of the infarcted papillary muscle to develop sufficient amounts of tension. (From Burch, DePasquale & Phillips. 1963. *Arch. Intern. Med.* 112:112)

of a unit volume of papillary muscle is probably greater than that of any other unit volume of myocardium. The great vulnerability of the papillary muscles to ischemic damage is demonstrated by the fact that one or both papillary muscles of the left ventricle showed evidence of recent or old infarction in 25 percent of 422 consecutive hearts studied at autopsy (Fig. 5) (5).

Since ventricular dilatation is common in patients with coronary (ischemic) heart disease, it is necessary to describe the influence of ventricular dilatation on papillary muscle function. As discussed above, during isovolumetric systole the mitral valve leaflets come into firm apposition to seal the A-V orifice. If the left ventricle is dilated, the papillary muscles migrate downward and laterally away from the A-V orifice. This centrifugal migration of the papillary muscles retracts the mitral valve leaflets into the cavity of the left ventricle, so that the mitral valve is incompetent during isovolu-

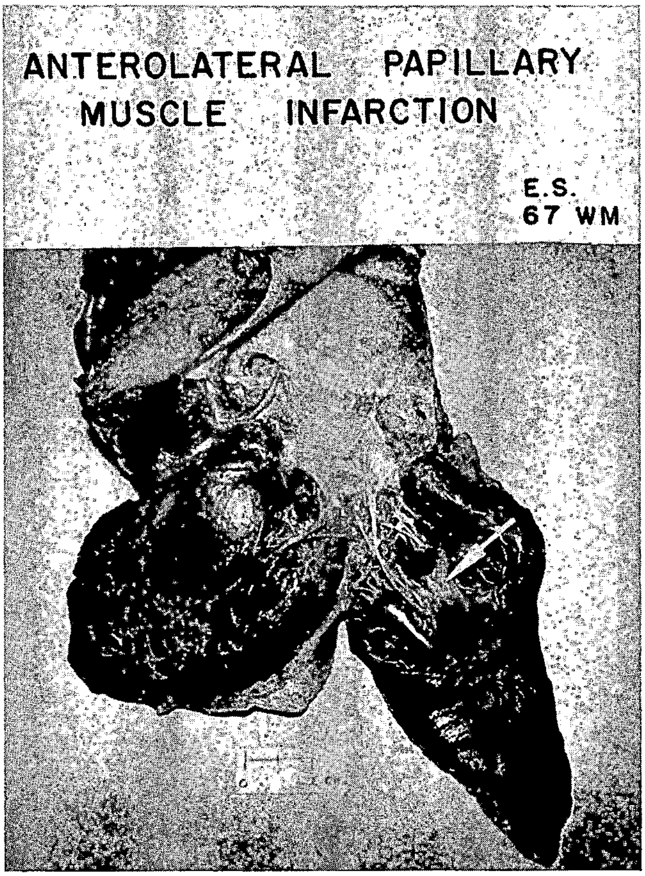


FIGURE 5. Heart of a 67-year-old patient with a large infarction of the anterolateral papillary muscle. (From Phillips, DePasquale & Burch, 1963. *Am. Heart J.* 66:338)

metric systole (Fig. 6). Furthermore, in the dilated heart, the papillary muscles must exert tension on the mitral valve leaflets more tangentially than normally, an obvious mechanical disadvantage, so that even if ventricular dilatation is associated with compensatory elongation of the chordae tendineae (11), mitral valve incompetence may occur.

CLINICAL MANIFESTATIONS OF PAPILLARY MUSCLE DYSFUNCTION

Papillary muscle dysfunction is associated with an apical systolic murmur, the characteristics of which depend upon the nature of the dysfunction. Theoretically, if a papillary muscle fails to contract adequately in a patient with a normal-sized heart, the resultant murmur should be delayed in onset and crescendo-decrescendo in quality (2). This is so because the

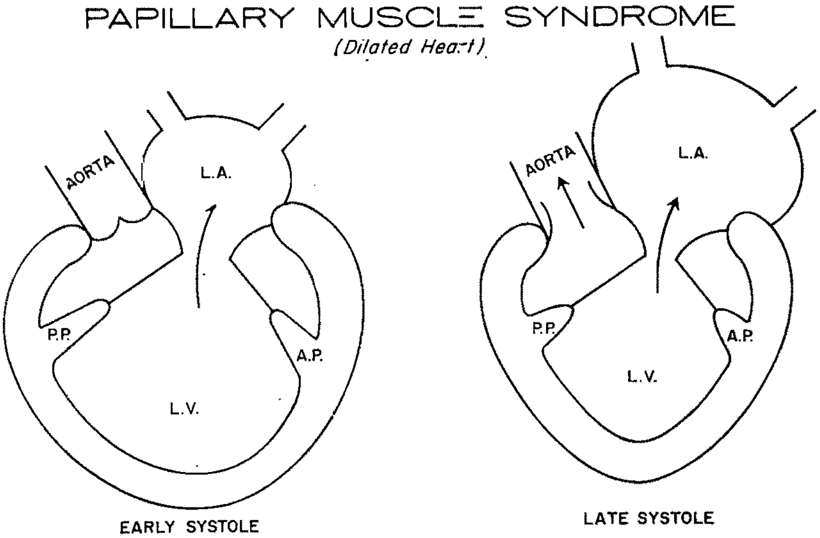


FIGURE 6. Diagrammatic representation of papillary muscle dysfunction due to ventricular dilatation. When the left ventricle is dilated the papillary muscles migrate centrifugally away from the A-V orifice, retracting the mitral valve leaflets into the cavity of the left ventricle so that the valve is incompetent even during the isovolumetric phase of ventricular systole. However, during ventricular ejection, the spatial relationships between the elements of the mitral valve apparatus may improve so that the associated murmur has a decrescendo quality. Thus, the murmur of papillary muscle dysfunction due to infarction or ischemia of a papillary muscle may be modified by left ventricular dilatation. (From Burch, DePasquale & Phillips. 1968. *Am. Heart J.* 75:399)

spatial relationships between the various elements of the papillary muscle apparatus are maintained during isovolumetric systole and only become altered during ventricular ejection. Although an apical diamond-shaped systolic murmur of late onset is sometimes observed in patients with ischemia and/or infarction of a papillary muscle (Fig. 7), it is more unusual for the murmur of papillary muscle dysfunction to start immediately after the first heart sound (Fig. 8). It is probable that failure of an ischemic papillary muscle to develop proper amounts of tension results in mitral incompetence even during isovolumetric contraction. In addition, because of intraventricular conduction delay, there may be failure to integrate contraction of the free wall of the left ventricle with papillary muscle contraction so that the mitral valve becomes incompetent, even though the distance between the base of the papillary muscle and the A-V ring is unchanged. Obviously, if the left ventricle is dilated and the mitral valve leaflets are retracted into the left ventricle, the murmur will be early in onset. However, under such circumstances the murmur may be decrescendo in quality as the spatial rela-

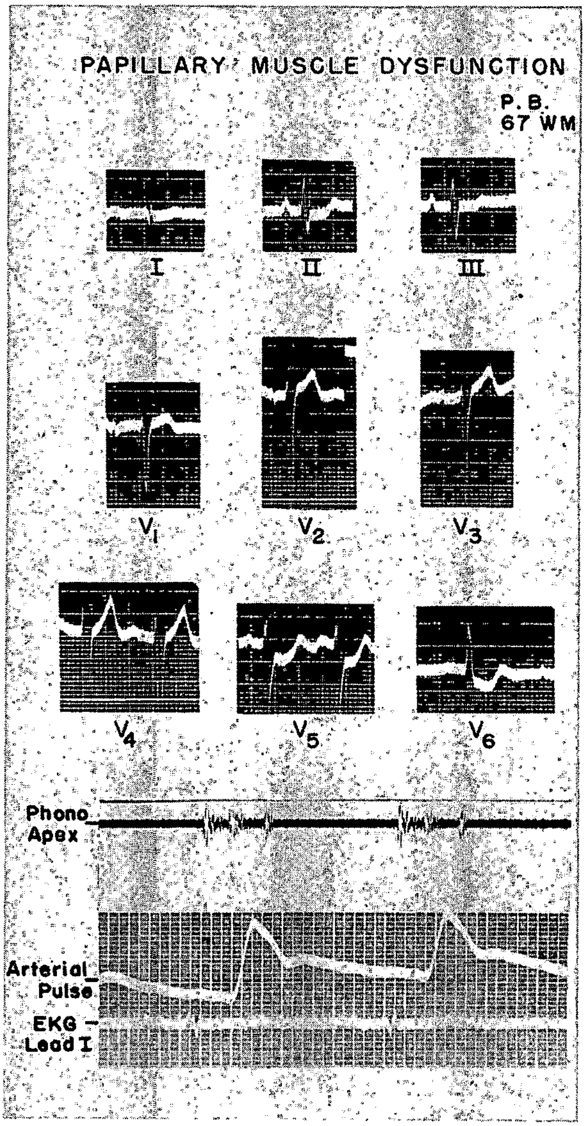
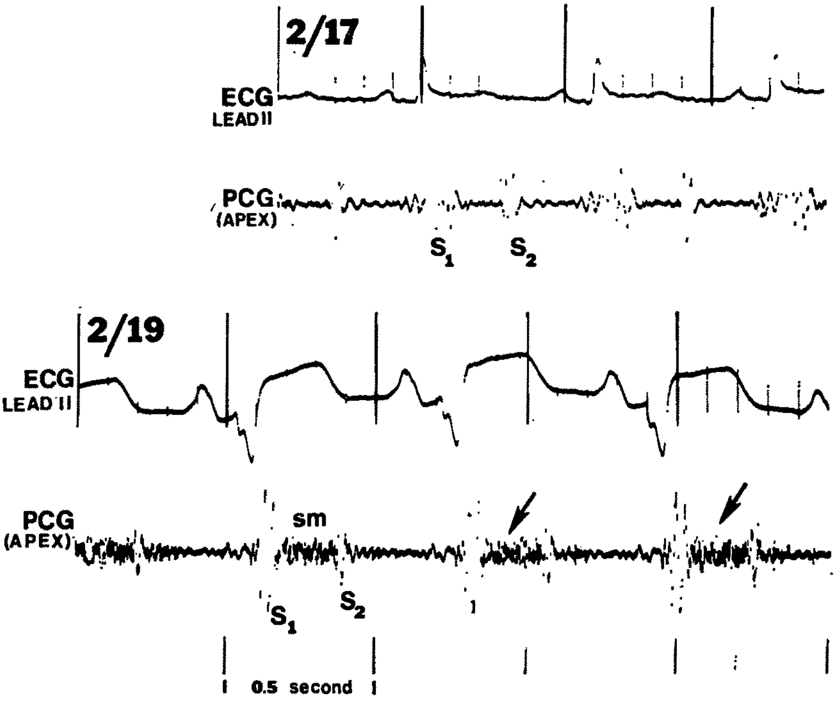


FIGURE 7. Electrocardiogram and phonocardiogram of a patient with papillary muscle infarction. The mid-systolic murmur is of late onset and diamond-shaped. The EKG shows Type III changes. Consult text. (From Phillips, Burch & DePasquale, 1963. *Ann. Intern. Med.* 59:508)



PAPILLARY MUSCLE DYSFUNCTION

FIGURE 8. Phonocardiogram of a patient with acute myocardial infarction. The patient was admitted to the coronary care unit on February 17 because of precordial chest pain. The EKG displayed ischemic changes and no murmur was audible. On February 19, the EKG displayed changes of acute myocardial infarction and a loud pansystolic murmur was audible at the apex.

tionships between the various elements of the mitral valve apparatus improve with ventricular contraction.

Regardless of the quality of the murmur, it is important to recognize that the sudden development of an apical systolic murmur in a patient with acute myocardial infarction or during an attack of angina pectoris may be caused by failure of a papillary muscle to contract. In this regard the sudden development of an apical systolic murmur in a patient with acute myocardial infarction is much more often caused by papillary muscle dysfunction than by a ruptured papillary muscle or perforated interventricular septum. Since ruptured papillary muscle or perforated interventricular septum are potentially amenable to surgery, differential diagnosis is of great importance. Aside from the characteristics of the murmur as described above, papillary muscle dysfunction is not usually associated with severe mitral regurgitation. Thus, whereas signs of left ventricular failure develop rapidly in patients with ruptured papillary muscle or perforated ventricular septum, they are unusual in patients with papillary muscle dysfunction.

As already indicated, papillary muscle function is dependent upon the adequacy of the blood supply to these structures. If papillary muscle ischemia is transient, as during an episode of angina pectoris, clinical evidence of mitral valve incompetence rapidly subsides as the papillary muscle regains the ability to contract. On the other hand, the clinical signs of mitral incompetence may regress slowly following myocardial infarction. Serial echocardiograms in patients with acute myocardial infarction and papillary muscle dysfunction have displayed day-to-day variations in diastolic slope velocity (12). Furthermore, the variations in diastolic slope velocity were associated with variations in the intensity of the apical systolic murmur (Fig. 9). These findings suggest that during the early stages of myocardial infarction the blood supply to the papillary muscles may be unstable. Thus, when papillary muscle blood flow improves, papillary muscle contraction also improves, diastolic slope velocity on the echocardiogram decreases and the murmur of papillary muscle dysfunction diminishes in intensity. On the other hand, when papillary muscle blood flow decreases, the velocity of the diastolic slope increases and the murmur of papillary muscle dysfunction increases in intensity (Fig. 9). It is probable that the same phenomenon occurs in other parts of the left ventricle following acute myocardial infarction, giving rise to electrically and mechanically "silent" but not biologically dead areas of myocardium (13).

The electrocardiogram may provide a clue to the diagnosis of papillary muscle infarction or ischemia (3). Three types of electrocardiographic patterns have been identified in patients with papillary muscle dysfunction. Type I consists of moderate depression of junction J, with a concavity-upward deformity of the ST-T interval (Fig. 10). Type II consists of slight-to-moderate depression of J with a prominent convexity-upward deformity of the ST-T interval and terminal inversion of the T wave (Fig. 11). Type III is characterized by marked depression of J, usually associated with a slight convexity-upward deformity of the ST-T interval (Fig. 7). When the electrocardiographic changes indicated above are displayed in leads I, aV_L, V_5 and V_6, involvement of the anterolateral papillary muscle is suggested, whereas changes in leads II, III, aV_F and V_1 through V_4 suggest involvement of the posteromedial papillary muscle. Overlapping of electrocardiographic signs indicates involvement of both papillary muscles. In general, type I changes are associated with chronic, fibrotic disease of the papillary muscle, whereas type II changes are associated with subacute disease, and type III with acute disease of the papillary muscle.

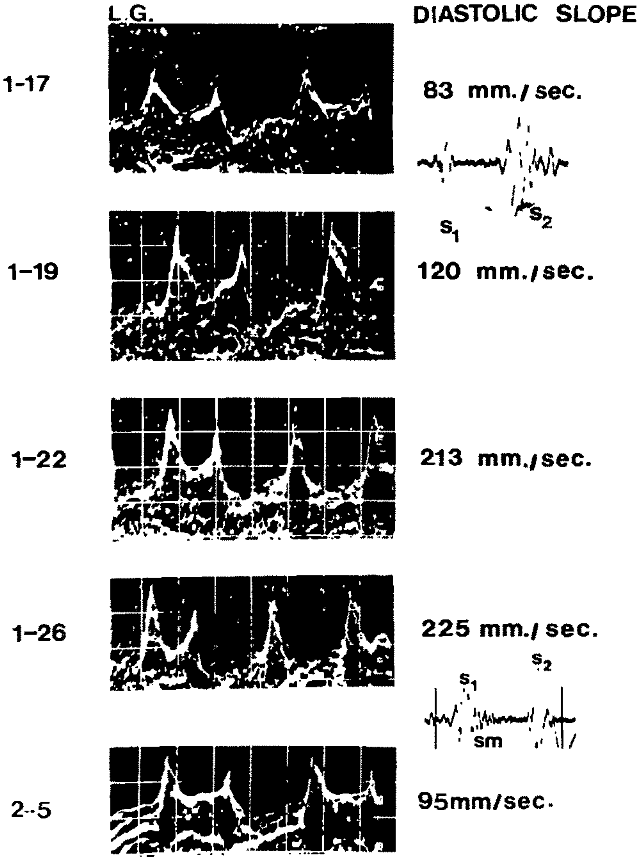


FIGURE 9. Serial echocardiograms of a patient with acute myocardial infarction. The changes in the velocity of the diastolic slope were considered to reflect variations in papillary muscle function. The numbers at the left represent the dates of the serial recordings.

DIFFERENTIAL DIAGNOSIS

The sudden development of an apical systolic murmur in a patient with an acute myocardial infarction may be due to ruptured papillary muscle, perforated interventricular septum, or papillary muscle dysfunction. The murmur of ruptured papillary muscle or perforated ventricular septum is pansystolic, usually associated with a thrill and heard over the entire precordium, whereas the murmur of papillary muscle dysfunction radiates to the axilla but is not heard over the entire precordium and is usually not associated with a thrill. In addition, left ventricular failure and signs of tricuspid

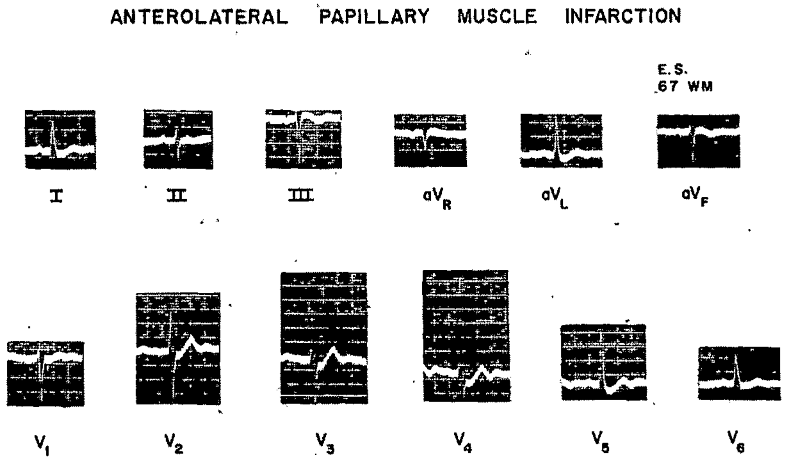


FIGURE 10. The electrocardiogram of a patient with papillary muscle dysfunction displaying Type I changes. Consult text. (From Phillips, DePasquale & Burch. 1963. *Am. Heart J.* 66:338.)

insufficiency develop rapidly in patients with ruptured papillary muscle or perforated ventricular septum. On the other hand, papillary muscle dysfunction rarely causes acute and severe left ventricular failure.

The murmur of papillary muscle dysfunction is often crescendo-decrescendo in quality and may, on occasion, radiate toward the base of the heart and even into the neck. Because of the ejection qualities of the murmur in some instances, it may be confused with the murmur of aortic stenosis when it is audible over the outflow tract of the left ventricle. Differential diagnosis depends upon such evidence as a history of rheumatic fever, exertional dyspnea, syncope, narrow pulse pressure, roentgenographic demonstration of a calcified aortic valve, and poststenotic dilatation of the aorta. Following administration of amyl nitrite, the murmur of papillary muscle dysfunction decreases in intensity whereas the murmur of aortic stenosis increases in intensity.

The murmur of rheumatic mitral regurgitation is pansystolic as in many instances of papillary muscle dysfunction, but, unlike the murmur of papillary muscle dysfunction, it is often associated with a thrill. A history of rheumatic fever, absence of electrocardiographic signs of papillary muscle infarction, and the age of the patient are of obvious value in differential diagnosis.

Apical systolic murmurs of late onset have received considerable attention recently (14, 15). These murmurs are frequently associated with one or

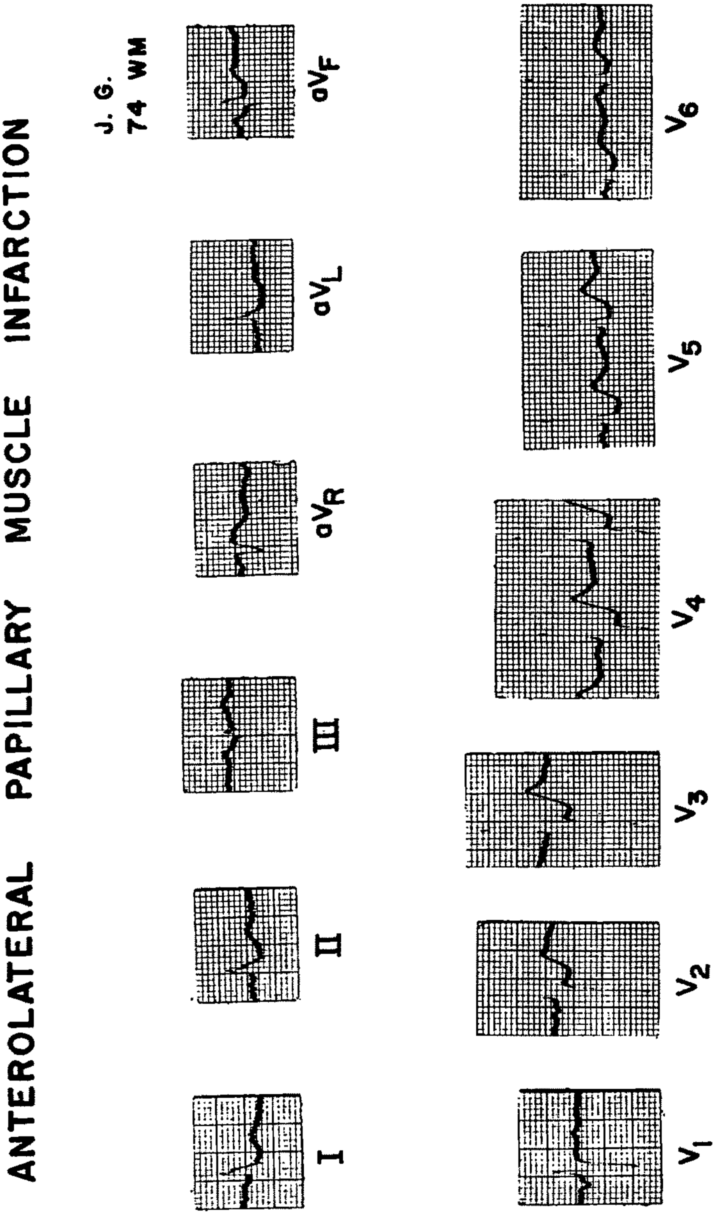


FIGURE 11. The electrocardiogram of a patient with papillary muscle dysfunction displaying Type II changes. Consult text. (From Phillips, DePasquale & Burch. 1963. *Am. Heart J.* 66:338)

more mid-to-late systolic clicks (nonejection type). Except for the so-called balloon mitral valve, the mechanism of these late murmurs has not been delineated, although abnormalities of the papillary muscles, chordae tendineae and/or valve leaflets have been suggested. These late systolic murmurs should not be confused with the murmur of papillary muscle dysfunction which is often but not always loudest in mid-systole rather than late systole and is usually not associated with systolic clicks. However, the murmur of papillary muscle dysfunction may vary considerably in its characteristics, depending upon the time course of the atrioventricular pressure differences and the associated time course of variations in partial closure of the mitral valve.

SUMMARY

Normal mitral valve function depends upon maintenance of the proper spatial relationships among the atrioventricular ring, the mitral valve leaflets and the papillary muscles and chordae tendineae throughout the cardiac cycle. A number of disease processes may result in papillary muscle dysfunction so that the normal spatial relationships between the various elements of the mitral valve apparatus are not maintained and mitral incompetence results. Ischemia or infarction of a papillary muscle and left ventricular dilatation are probably the most common causes of papillary muscle dysfunction.

Infarction or ischemia of a papillary muscle may render the muscle partially or completely incapable of contraction. During isovolumetric contraction the proper spatial relationships between the elements of the mitral valve apparatus are maintained. However, during ventricular ejection, because of the failure of the papillary muscle to shorten, the chordae tendineae slacken, allowing a portion of each mitral valve leaflet to evert into the left atrium, resulting in mitral incompetence. Although the murmur associated with papillary muscle dysfunction may be delayed in onset, it usually begins with the first heart sound. It is likely that, even though the proper spatial relationships between the elements of the mitral valve apparatus are preserved, the infarcted or ischemic papillary muscle does not develop sufficient tension to maintain mitral valve competence. With left ventricular dilatation, the papillary muscles migrate centrifugally away from the atrioventricular ring retracting the mitral valve leaflets into the cavity of the left ventricle to produce mitral incompetence and an associated murmur which begins with the first heart sound.

The auscultatory and electrocardiographic findings associated with ischemia and/or infarction of a papillary muscle, together with the clinical setting, constitute a fairly typical syndrome which is being recognized with increasing frequency.

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LATE COMPLICATIONS OF HEART VALVE REPLACEMENT

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Valve replacement by prosthetic device or graft has become an accepted method of surgical approach in the treatment of valvular heart disease during the past decade. Reasonable operative risks have been achieved by improvement in surgical technics and heart-lung bypass procedures. Control of infection, heart block, coagulation problems, and the recognition of the various postperfusion syndromes have helped reduce early postoperative mortality.

The effect of valve replacement on the natural course of heart disease is dependent upon the status of the pre-existing myocardial reserve, the degree of hemodynamic improvement accomplished, and the reversibility of functional disability. The etiology of the underlying heart disease—inflammatory, congenital, infectious, or degenerative—plays a part. Long-term results are further influenced by complications directly related to the prosthetic device.

Poor mechanical performance and durability has caused the abandonment of flexible leaflet artificial prostheses. Most surgeons now use a variety of rigid artificial valves either of the caged-ball or low profile-disc type. Others are substituting homologous or heterologous valve grafts for the diseased tissue. The late complications of these procedures will be considered separately.

PROSTHETIC HEART VALVES

THROMBOEMBOLISM

Thromboembolism is one of the most significant and serious late complications of the use of valve prostheses (1–4). A review of 426 patients with caged-ball insertions at the Mayo Clinic between 1960 and 1965 (4) revealed an incidence of postoperative embolism in 33 percent of mitral, 31 percent of aortic, and 23 percent of combined valvular replacements. The overall mortality associated with these embolic episodes was 15 percent. Another series of 438 patients (1) operated on from 1961 to 1968 had 66 late deaths of which 16 were attributable to thromboembolic complications.

Atrial fibrillation has been associated with a significant increase in the incidence of emboli after valve replacement (4–6). Neither the presence of left atrial thrombus at the time of surgery nor a history or prior embolization increased the risk of postoperative emboli. Furthermore, there was no reduction in the occurrence of embolism with the passage of time.

The almost equal incidence of mitral and aortic-replacement embolization in the Mayo Clinic series (4) was not in accord with others who found fewer emboli with aortic than with mitral prostheses (2, 5–9). Because of the low frequency of emboli following aortic valve surgery in their experience, neither Effler (2) nor Cooley (8) used anticoagulants. Yeh et al (5) found warfarin sodium effective in the mitral group and had no emboli in their 36 aortic replacements with or without anticoagulation. In the Mayo Clinic study, the high incidence of emboli following aortic replacement was reduced strikingly with anticoagulant therapy. Most agree, however, that differences in results between inadequate levels of anticoagulation and the nonuse of anticoagulants are negligible (4–6).

The most frequent sites of embolization, cerebral vessels (4, 5, 10), had a lower mortality than the less common but more clinically severe coronary embolism. In part, the relatively high incidence of cerebral emboli may be due to easier recognition of these lesions. On the other hand, clinical evidence of systemic emboli during life was far less common than the postmortem finding of thrombi on the prosthetic valves. At the National Heart Institute thrombi were present at necropsy in 38 of 49 patients (11). A similar high frequency was encountered on aortic, mitral, or multiple-valve prostheses. Only 4 of these 38 had clinically diagnosed antemortem systemic emboli. Nineteen patients with thrombi had received warfarin sodium and 5 without thrombi had been on anticoagulant therapy. Valve function had been impaired by the thrombus in 13 instances. The thrombi were attached to the metallic parts of the valves extending up the struts from their origin at the fixation rings.

The association of thrombus formation with the exposed metallic parts of the prostheses provoked interest in reduction of thrombogenesis by tissue encapsulation of the cage (12–14). Experience with a knitted Teflon totally covered cage in a two-year period from 1967 to 1969 has reduced significantly the incidence of clinical embolization (15), but until long-term observations are complete the use of anticoagulation generally still is recommended.

HEMOLYSIS

Hemolysis resulting from mechanical injury to erythrocytes by prosthetic valves seemed greater as the valves were placed closer to the left ventricle (16, 17). Increase in cardiac output during exercise hastened the rate of hemolysis (18). Hemosiderinuria may lead to an iron deficiency anemia with a high output state, further accelerating the hemolysis (19). Postalimentary lipemia has been shown to augment the susceptibility of red blood cells to mechanical disruption (20). Other factors include decreased erythrocyte survival time (21) and reduced serum haptoglobin in the nonanemic patient (22). The presence of endothelium on the fixation ring may be a deterrent. Hemolysis has been controlled when exposed Teflon is covered by endocardium (23).

Postoperative hemolysis is most likely to occur in the presence of significant paravalvular leaks (1, 24–26). These leaks resulted from broken or pulled-through sutures or holes between the valve ring and basal tissues. Calcification seemed to be of more importance in contributing to the leaks in the aortic rather than in the mitral area. Surgical correction of the leaks was helpful in reducing red blood cell destruction.

Hemolysis was greater with aortic prostheses presumably because of the greater velocity of blood in this area. Despite the development of the Davila low-profile mitral prosthesis with a Dacron-covered sewing ring to eliminate a thrombogenic interface (12), accelerated hemolysis following mitral replacement with this device has discouraged its continued use (27). Most patients with ball valves have some accelerated hemolysis but the majority of these have sufficient bone marrow compensation to prevent the development of anemia. Accordingly, hemolysis is not often a serious or uncontrollable problem. Medical management with folic acid and iron usually is satisfactory and repair of perivalvular leaks is dependent upon their hemodynamic significance.

VALVE DYSFUNCTION

Degeneration of the silicone rubber poppet, ball variance, is being reported with ever-increasing frequency. There has been fissuring (28), fracture (29), shrinking, swelling, softening, and change in color (30). Reasons for ball variance are not known but possible factors include impurities in the preparation of the silicone, drug diffusion, and the absorption of blood lipids. Malposition of the prosthesis, a too-tight fit, or perivalvular leaks may play a role. Physical stress in the high-pressure, high-velocity aortic area probably is important in causing ball degeneration since mitral poppet failure is a rarity. Only recently two instances of swollen poppet of the Smeloff–Cutter mitral prosthesis (31) and one failure of a Starr–Edwards mitral ball were described (32). Impaired function of the newer cloth-covered Starr-Edwards mitral prosthesis (33) and of the Smeloff–Cutter valve (34) also has been reported.

Death may be caused by systemic embolization of the entire ball (35–37) or obstruction of the valve orifice itself by swelling of the poppet (38). In a necropsy study of 47 late postoperative deaths (39) aortic ball variance was noted in all 12 patients surviving more than 24 months. In 11 of these the degeneration was severe. None of the 23 patients who died less than 24 months after aortic valve replacement had ball variance. Degeneration of the poppet was not noted in the 12 patients with mitral prostheses alone nor in the 9 with both mitral and aortic replacement. Systemic embolization of ball fragments following fracture of a Starr–Edwards aortic poppet has occurred with an unusual foreign-body giant cell reaction in the liver and spleen (11, 40). Many of these cells containing asteroid bodies were located in areas adjacent to nonbirefringent yellowish-brown particulate matter, presumably related to fragmentation of the prosthetic ball. Introduction of a cobalt, chromium, molybdenum, nickel alloy, Stellate 21, hollow poppet in 1967 (14) may eliminate the ball variance noted with the earlier use of silicone rubber.

Although ball variance is rare in the mitral position other mechanical failures have been encountered. The use of an open-cage Cutter ball valve caused engagement of the wall of the ventricle or a papillary muscle by a strut (41). This caused restriction of the movement of the ball and laceration of the myocardium. Mitral insufficiency in four patients with caged-lens mitral prostheses resulted from failure of valve closure by eddy currents or the jet of coexisting aortic regurgitation (42). Fibrinous encroachment extending from the fixation ring caused mitral stenosis in three patients with Kay–Shiley disc valves (43). In one patient there was ingrowth of fibrogranulomatous tissue along the mural surface as well.

The recognition of prosthetic valve malfunction has been a continuing problem in the management of the postoperative patient. Timing of auscultatory events, changes in the intensity and quality of the prosthetic sounds, variation in the characteristics of murmurs and the appearance of new ones have been documented both clinically and phonocardiographically (44, 45). Radiographically and with the use of cineangiography, filling defects of the wall, obstruction by clot, and abnormal poppet motion or movement of the cage may be detected (46). Abnormal echo waves may be recorded by ultrasound (47–49). The development of new symptoms after a patient makes a satisfactory initial recovery should provoke an investigation into the proper functioning of the valve. Sudden appearance of valvular regurgitation, congestive heart failure, embolization, and hemolytic anemia have been associated with prosthetic valve dysfunction.

INFECTION

Postoperative bacterial endocarditis due to contamination by organisms introduced at the time of surgery significantly has been reduced by the prophylactic use of antibiotics (50–52). Late development of infection on a prosthetic valve usually is secondary to an intercurrent bacteremia such as occurs following dental extractions. The pathological lesions with extension into adjacent tissue and abscess formation make treatment difficult (53, 54). Successful replacement of infected prostheses is being accomplished (6, 55).

Extension of infection may cause atrioventricular or intraventricular conduction defects. Fatality in four patients with aortic prosthetic infection was caused by aortic regurgitation and heart failure resultant from detachment of the valve (11). Separation of the attachment of the mitral valve was noted in one case; two others had infected atrial thrombi with obstruction of the mitral orifice.

Most of the reported cases of bacterial endocarditis were caused by *Staphylococcus, Serratia, Proteus, Pseudomonas,* and various fungi. Of great interest has been the occurrence of endocarditis caused by organisms considered to be nonpathogenic in man. *Corynebacterium xerosis* endocarditis has been proved in two cases with Starr–Edwards prosthetic valves (56). In one patient the onset of endocarditis was not documented until 14 months postoperatively, making introduction at the time of surgery most unlikely. A cooperative statistical study of 27 different surgical groups (57) included 3620 patients in whom one or more valves were replaced. Infection accounted for 2 percent of the nonfatal complications after valve replacement.

PARIVALVULAR LEAKS

Improved operative technics, particularly in regard to increasing the multiple fixation points of the valve ring, have been effective in reducing parivalvular leaks. Although this problem, recognized early in the postoperative period, may be corrected by reoperation, late deaths due to perivalvular fistulae were observed in 7 of 49 patients coming to necropsy at the National Heart Institute (11). In three with isolated aortic prostheses, the leak was determined prior to death. This diagnosis was established in only two of the remaining patients with mitral area fistulae. In two patients with combined valvular replacements, perivalvular leaks were present only in the mitral area. None of these seven patients had bacterial endocarditis although detachment of the prosthesis was frequent in fatal postoperative infection.

SECONDARY PATHOLOGICAL CHANGES

Left ventricular endocardial fibroelastosis following valve replacement has been a cause of death by heart failure in the late postoperative period (58). This was not seen in patients dying during the first month after surgery but was present to some degree in all 22 patients with late deaths following Starr–Edwards mitral replacement (11). In 17 of these, the fibrous endothelial thickening involved large areas of the left ventricle as well as the stumps of the amputated papillary muscles and sites of drain insertions. It was most extensive in two patients with both aortic and mitral prostheses but was not found in any of the patients with isolated aortic replacements. Left atrial endocardial thickening was seen in all of the mitral insertions but did not contain an elastic tissue component, in contrast to the left ventricular changes. The fibroelastosis occurring in the left ventricle was thought to be secondary to turbulence of blood flow caused by the up-and-down motion of the ball and the fact that blood flows around the poppet in closer proximity to the endocardial surface rather than centrally through the valve, as in the normal situation. Moderate endocardial fibroelastosis of the left ventricle was noted in one patient who died 21 months after Kay–Shiley disc mitral replacement (59). This surgical group speculates that this complication may be occurring in three of their surviving operative patients. The impression of acquired myocardial restriction in these cases is supported by angiographic evidence of poor myocardial contractility and elevated left ventricular end-diastolic pressures.

Fibrous proliferation caused by turbulent flow after aortic replacement occurs in the aortic root and this intimal thickening may involve the coronary arteries (11). All of the 37 patients with Starr–Edwards aortic prostheses had some intimal proliferation of varying severity, either diffuse or focal in the aortic root. In 18, this process included one or both proximal coronary arteries or ostia. The degree of narrowing of the coronary arteries was not significant in most instances but was of obvious importance in a few cases.

MORTALITY

Operative deaths in most large series of prosthetic valve replacements varied between 11 and 20 percent for aortic, 9 and 24 percent for mitral, and 13 to 32 percent for multiple-valve insertions (11, 60–62). Late deaths added another 10 to 15 percent to this mortality, placing the one-year survival rate in the 70-percent range. A three-year follow-up of 100 patients with aortic valves replaced by Effler (61) revealed only 53 percent still living. In a large series of 1323 patients undergoing surgery at the Mayo Clinic (4), only 40 percent of the aortic and 50 percent of the mitral replacements were alive at the end of five years. A six-year follow-up of Cooley (63) found 68 percent of 2097 patients surviving with various prosthetic valve insertions.

Since fatal late complications such as thromboembolism, mechanical failure of the valve, and infection accounted for only about 11 percent of the deaths in the first year, the remainder were either unexplained sudden exitus, unrelated events, or deterioration of the myocardium. The variability of mortality rates in part must be due to patient selection and the degree of existing myocardial degeneration.

HOMOGRAFT AND HETEROGRAFT HEART VALVES

The first successful use of a homograft aortic valve in the descending aorta of man was accomplished in 1956 (64). This cadaver aortic valve still was functional six years later when eight additional cases were described (65). The possibility of insertion of these valves in a subcoronary position was established (66) and then applied in humans (67, 68). Six years of clinical use in various medical centers now permit some evaluation of the durability of homograft valves and the complications attendant upon its use in replacement of diseased aortic valves.

Aortic valve homografts have been used in reconstruction operations for hypoplasia of the right ventricular outflow tract (69) and as replacements for the tricuspid and mitral valves as well (70, 71). When used in the atrioventricular position the grafts are fixed to a fabric-covered metal frame for better stability (72). Experience still is too limited to assess adequately the use of aortic homografts in other than aortic valve replacements.

The easy availability of various animal valves has prompted their substitution for human valves in recent years. Since the first heterologous graft replacement of an aortic valve in a subcoronary position in 1965 (73), pig, calf, and sheep tissues have been used in the aortic (74–77) and mitral positions (75, 78–80). Pathological examination of removed grafts reveals that both heterologous (80) and homologous valves (81) act merely as an inert framework for replacement by host tissue. Complications with heterograft and homograft valves appear to be similar but use of animal valves in humans to date is too limited to warrant definitive conclusions.

HEART MURMURS

The late development of the murmur of aortic regurgitation appeared to be the commonest complication encountered in all large series of homograft aortic valve replacements (82–84). This was more frequent when aortic regurgitation was present preoperatively (83). The importance of proper placement in the avoidance of the development of this murmur was stressed in a review of the first 50 cases of aortic homograft valve replacement at the Mayo Clinic (85). An aortic diastolic murmur was noted at the time of discharge in 17 percent of these patients but was not considered hemodynamically significant. During the first postoperative year 44 percent of the 36 patients available for follow-up had audible aortic diastolic murmurs. Others noted an incidence as high as 80 percent in the development of late postoperative diastolic murmurs (86).

Aortic systolic murmurs were present after surgery almost universally. They were usually of low intensity and unchanged over the periods of observation unless malfunction of the valve developed. Despite the frequency of postoperative systolic murmurs only occasional significant differences in systolic pressure between the left ventricle and aorta were observed during cardiac catheterization (83, 86–88).

AORTIC REGURGITATION

Valve dysfunction with aortic regurgitation may occur secondary to parivalvular leaks at the line of attachment of the graft, an early complication reduced by better surgical technics. Later dehiscence of the graft has been noted at the time of reoperation (82). Malplacement of the valve with a central leak causing valvular insufficiency is another problem in operative procedure that is becoming less common (84).

In one series, eight of ten patients developing aortic insufficiency had rupture of valve leaflets (89). This was the most common cause for late aortic regurgitation, occurring between 6 and 18 months but rarely more than two years after surgery (84). The ruptured cusps caused progressive and severe disability but not sudden death. Calcification of leaflets seemed to be a factor in some instances (83) but was rare and never significant in less than three years after surgery (84). Between three and six years postoperatively, calcification is uncommon and had not occurred when the homograft was collected with strict sterile precautions and placed in Hank's solution, a combination of antimicrobials. This is in accord with the close association of calcification with infection in necropsy material (89).

INFECTION

Postoperative endocarditis, either bacterial or fungal, was encountered in small numbers (82–84). In the large experience of Barratt-Boyes with 590 operations (84), infection was uncommon and attributed to the procedures of achieving adequate sterility.

THROMBOEMBOLISM

Systemic thromboembolism was uncommon in the largest series of homograft valve replacements despite the lack of anticoagulant therapy (82, 84). In others (83, 85, 86, 90) there were no clinically apparent embolic phenomena. During a six-year follow-up of 532 patients surviving surgery, Barratt-Boyes encountered no deaths from embolization.

MORTALITY

Early postoperative mortality following homograft aortic valve replacement varied from 6 to 20 percent (82, 85, 91), with later deaths creating a cumulative mortality of 32 percent (91) and 34 percent (82). In the largest series (590 patients) with the longest follow-up (six and one-half years) Barratt-Boyes, one of the pioneers in the development of this procedure, reported a hospital mortality of 9.8 percent and a late mortality of 4.9 percent, secondary to valve failure. Although statistical comparisons with prosthetic valve replacement cannot be made because of much more frequent and longer experience with artificial devices, it would appear that the late risks of valve grafts will not exceed those of prosthetic valve insertion.

SUMMARY

During the past decade valve replacement has represented a significant advance in the management of valvular heart disease. Improvement in prosthetic devices and experience with homologous and heterologous valves has permitted the clinical use of these technics in an increasingly larger patient population.

Thromboembolism still remains a troublesome complication in the late follow-up of patients with prosthetic valves. Mechanical failure of the prosthesis, hemolysis, and infection are other problems associated with the presence of artificial valves. The late development of left-ventricular fibroelastosis in patients with mitral prostheses deserves further study.

The use of human or animal valve grafts largely obviates the problem of

thromboembolism. Aortic regurgitation, resulting from disruption of the valve cusps, is the most serious complication of this procedure.

An operative mortality of about 10 percent or less has been achieved by most surgical groups. However, with the later complications of valve replacement and the effects of the underlying heart disease itself, a survival rate of 50 to 70 percent in these early postoperative years is being reported. Under the circumstances asymptomatic patients are not considered candidates for valve surgery. In patients with functional disability a reasonable question would be whether the patient has a 50–70 percent chance of being alive in one year without surgery. In the future, better understanding and resolution of the complications attendant upon valve replacement may further reduce the mortality enough to permit surgery in patients with anticipated severe disability before it actually is present. Allowing the surgeon to operate upon a better-risk patient may reverse some hemodynamically damaging valvular deficiency before irreversible myocardial deterioration has occurred and further reduce the late mortality.

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ABNORMALITIES IN CARDIAC CONTRACTILITY ASSOCIATED WITH LOCALIZED MYOCARDIAL DAMAGE

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Myocardial asynergy, or regional deviation from coordinated contraction of the ventricular wall, was first studied by Tennant & Wiggers (1) who noticed localized outward bulging of the canine ventricle one minute after a coronary artery was ligated. With the kinetocardiograph, Harrison (2) demonstrated and classified chronic asynergy in humans with coronary artery disease and suggested that regional abnormalities might cause generalized disturbances in ventricular function. Recent studies have confirmed this suggestion and, by combining cineangiography with measurements of flow and intracardiac pressure, have shown how chronic asynergy alters both the internal mechanics and the external work of the heart (3–5). The information obtained from these studies is of particular relevance in helping to decide which patients with chronic asynergy should have corrective surgery. Acute regional asynergy may occur with angina or acute myocardial infarction and may also produce generalized alterations in cardiac performance.

NORMAL SEQUENCE OF CARDIAC ACTIVATION AND CONTRACTION

Depolarization is propagated rapidly through the endocardium and free wall. Regional differences in activation time are normally small (6, 7) and probably cause only minimal mechanical asynchrony (8). The Purkinje system and ventricular depolarization are resistant to hypoxia and it is unlikely that the changes in contraction associated with brief ischemia reflect a failure in conduction. However, gross changes in the overall sequence of activation, as with ventricular premature contractions, can cause significant regional asynergy (5). Sarcomeres which are activated early may stretch neighboring inactive segments beyond the optimal length for force generation, making them unable to oppose intraluminal pressure (9). The heart responds functionally as a syncitium; excitation spreads rapidly between cells across the low-resistance intercalated discs and quickly enters cells via the transverse tubular system, an extension of the sarcolemma. The lateral sacs of the sarcoplasmic reticulum lie adjacent to the transverse tubules and are rapidly depolarized. Bound Ca^{++} is released and penetrates via the reticular network to the active sites on the myosin crossbridges and actin filaments (10, 11). Extracellular Ca^{++} may also be pumped in by an active transport mechanism. Calcium is thought to activate contraction by binding reversibly to troponin, thereby causing tropomyosin to undergo a conformational change which permits the active sites to interact (12); Ca^{++} also stimulates myofibrillar ATPase. In general terms, the intensity of the active state, or the number of active sites which interact to generate force and shortening, seems related to the degree of Ca^{++} release (11). Relaxation may involve uptake of Ca^{++} by the repolarized sarcoplasmic reticulum or perhaps by mitochondria (13). The effect of hypoxia on excitation-contraction coupling is not clear. Sarcoplasmic reticulum isolated from areas of myocardium which were ischemic for 60 min showed a reversible decrease in ability to take up Ca^{++} (14). Hypoxia might also affect excitation-contraction coupling by decreasing active Na^{+}-Ca^{++} exchange at the myocardial cell membrane, or by causing accumulation of H^{+}, which may compete for the binding sites on troponin (12).

Energy for contraction is provided by the terminal phosphate bond of ATP, which forms a reversible linkage between myosin crossbridges and actin filaments; hydrolysis of ATP may permit the sliding movement of the myofilaments. Adequate levels of ATP and a reserve of readily available high-energy phosphates as creatine phosphate (CrP) depend normally on oxidative phosphorylation. With regional ischemia, anaerobic pathways for the generation of energy are rapidly activated but these are inadequate to maintain effective contraction. Because of problems in tissue sampling, it is not entirely clear whether contractile failure due to ischemia precedes or follows depletion of CrP and ATP (15, 16).

GEOMETRIC CHANGES IN THE NORMAL CONTRACTING VENTRICLE

The left ventricle (LV) is not entirely homogenous in its structure or mechanical behavior, so some degree of asynergy exists normally. Ventricular muscle consists of twisting syncitial fibers which form bundles oriented into major internal and external helices (17). Although there are no discrete muscle layers, deep fiber bundles generally lie at right angles to superficial bundles (18) and may act functionally as constrictors which direct force inwardly (19). Sarcomere length in the midwall shortens with systole from an average of 2.07 μ to 1.81 μ, but there is considerable normal "dispersion" in length which, if exaggerated by dilatation, might cause regional variations in contractile strength (9). Regional differences in the rate at which the LV wall shortens have been demonstrated in dogs with implanted strain gauge arches (20).

In general, with isovolumic contraction there is slight shortening of the base to apex length and an increase in internal circumference, producing a change in ventricular conformation from an ellipsoid to a sphere; average wall thickness increases by 6 percent. With ejection, the long axis shortens by an average of 5–7 percent and the internal circumference decreases by

22–30 percent; wall thickness increases by an additional 9 percent (21–24). These changes vary considerably with changes in ventricular filling or inotropic state. It is difficult to estimate stresses within the contracting LV wall because of the dynamic changes in intraluminal pressure, or force, and wall geometry. If the LV is considered as a thin-walled sphere, the Laplace relationship can apply (Stress $= PR/2h$, where $P =$ pressure, $R =$ internal radius, and $h =$ wall thickness). As R normally decreases during ejection and h increases, wall stress decreases; obviously, wall stress will be greater if R is larger and wall thickness is less. More accurate estimates of instantaneous wall stress can probably be made if the ventricular shell is considered as an ellipsoid of revolution which, during systole, decreases its circumferential curvature and increases longitudinal curvature to become more spherical (21).

METHODS OF MEASURING MYOCARDIAL PERFORMANCE

The performance of the ventricle as a pump is expressed in the ventricular function curve, which relates end diastolic volume or pressure to ventricular work. This Starling curve has its structural basis in the relationship between the initial length of the component sarcomeres in muscle and the development of active tension (25). Stroke index and ejection fraction (stroke volume/end diastolic volume) and their response to exercise provide an overall estimate of the hydraulic function of the pump. Left ventricular end diastolic pressure (LV_{EDP}) is limited as an index of ventricular performance since it can be affected by changes in the compliance of the ventricular wall.

The contractile state of the myocardium is reflected in the inverse relationship between force (or load), and the velocity of shortening, or the force-velocity curve. In the three component model for muscle suggested by A. V. Hill, a contractile element (CE) is connected to a series elastic element (SE) and both components are linked to a parallel elastic component (PE). The CE resides in the contractile proteins, but the other two components have no clear anatomical localization. At rest, or in diastole, the PE supports resting tension. During isovolumic contraction the CE shortens by 4–5 percent and stretches the SE at a velocity which is inversely proportional to the stiffness of the SE. With isometric contraction, or ejection, (26), the CE shortens another 10–15 percent and the SE begins to recoil.

The maximum velocity at which unloaded muscle shortens, or V_{max}, theoretically reflects the maximum intensity of the active state, or the inherent contractility of the CE, and is influenced only by inotropic factors (25). The validity of this concept for heart muscle has been questioned (27), but the measurement is useful. There have been many approaches to the estimation of V_{max} by physiologic measurements in the intact heart. The velocity of shortening of the CE can be calculated at various loads and the resulting force-velocity curve is extrapolated to zero load, yielding V_{max}. This method entails instantaneous estimates of wall stresses and of the velocity of shortening of the midwall circumferential fibers (V_{CF}) (25, 28–30). V_{CF} can be measured directly from cineangiograms or calculated from the ejection fraction, using formulae for the volume of ventricular models. However, these methods al. assume uniform movement and thickness of the ventricular wall and are inaccurate if there is significant asynergy.

In practice, reasonable approximations of V_{max} can be made from the first derivative of the ventricular pressure curve by determining peak isovolumic dp/dt, or the peak rate of pressure development. Various normalizations of peak dp/dt for changes in preload and afterload have been proposed for a more accurate index of contractility (29, 31, 32). Estimated V_{max} correlated well with peak isovolumic dp/dt normalized by dividing by peak isovolumic pressure (dp/dt/PIP) (30) but valid comparisons between patients may require corrections for wall thickness and diastolic volume (29). The second derivative of the ventricular pressure curve, d^2p/dt^2, or the acceleration of pressure development, has been suggested as a more sensitive index of ventricular contractility (33), but its usefulness needs study.

VENTRICULAR CONTRACTILITY IN HEARTS WITH ASYNERGY

Experimentally produced aneurysms of the LV in dogs caused acute depression of ventricular function curves which improved if displacement of blood into the aneurysms was prevented (34, 35). There has been no report of studies with chronic replacement of graded areas of the LV with nonexpansile patches, but the combined effects of an increase in cardiac radius, a loss of contractile elements, and a change in the SE would be operative.

Coronary artery disease is the most frequent clinical cause of regional asynergy leading to impaired cardiac contractility; post-traumatic aneurysms can cause heart failure but are more prone to rupture (36). Theoretical considerations first suggested that the depth rather than the surface area involved in an infarction was of greatest importance in determining whether an aneurysmal bulge would occur (37). However, a mathematical analysis using a thin-walled sphere as a model of the human ventricle predicted that when the percentage of inactive or aneurysmal wall surface exceeded 20 percent, systolic shortening of the surviving muscle would have to exceed normal physiologic limits to maintain stroke volume (38).

The first detailed clinical analyses of the effects of asynergy were made in Gorlin's laboratory and substantiated the mathematical predictions (3–5). The hemodynamic findings in a large group of patients with coronary artery disease and definite LV asynergy on angiography were compared with the findings in patients with comparable degrees of coronary artery disease but no asynergy. Both groups showed impaired function when compared to normal controls. The group with significant asynergy had a higher incidence of congestive heart failure and lower mean values for stroke index and systolic ejection rate. LV_{EDP} was elevated in both groups, but only patients with asynergy showed increases in mean values for LV end diastolic volume (LV_{EDV}) and a significant decrease in mean values for the rate of rise of isovolumic pressure ($\Delta p/\Delta t$), a calculated approximation of true dp/dt. Heart size and LV_{EDP} were often normal with asynergy despite the presence of low stroke output and clinical heart failure.

The dynamic behavior of the ventricular wall during systole was examined by superimposing calibrated, life-sized silhouettes of successive film frames and measuring serial changes in multiple plotted axes. Local areas of systolic asynergy were classified as showing dyskinesis, or paradoxical expansion, akinesis, or absence of motion, asyneresis, or decreased motion, and asynchrony, or a disturbed sequence of motion. Several of these varieties of asynergy were often present in one heart. Although the term aneurysm derives from the Greek word for "broadening," it is used to describe both dyskinetic and akinetic areas.

Dyskinetic areas can interfere with cardiac performance by several mechanisms. The contribution of the damaged area to overall ventricular work is lost and the remaining muscle must shorten more; paradoxical movement of the wall or an aneurysmal sac will permit displacement of blood from effective forward flow, as in mitral insufficiency; the scarred area is usually slack and behaves as a compliant SE which slows the development of force by surviving adjacent muscle.

Akinetic or asyneretic areas may be rigid scars or may contain active contractile units which generate sufficient local force to prevent paradoxical movement, but make no contribution to effective ventricular work. The remaining muscle shortens more to maintain stroke volume; the scar may add a more compliant or a more rigid SE, depending upon its elastic properties during systole. Asynchrony can affect ventricular performance by all of these mechanisms since the regional activation, force generation and development of active stiffness are out of phase with the remaining muscle.

Among asynergic hearts, akinesis was more frequent than dyskinesis (4). Angiographic estimates of the size, location, and type of asynergy correlated well with findings at surgery and autopsy. The location generally coincided with angiographic evidence of coronary disease and with electrocardiographic signs of infarction or of the repolarization abnormalities characteristic of aneurysms. Apical and anterior regions were more often involved since these areas have thinner walls and are more easily outlined by single-plane angiography. Regional metabolism of lactate was often abnormal, but no more than in hearts with no detectable asynergy.

With localized asynergy, the contribution to stroke volume made by uninvolved areas was increased. If large volumes were displaced into dyskinetic areas or if akinetic areas comprised more than 20 per cent of the surface area, there were significant increases in LV_{EDV} and LV_{EDP}. Compensation by the Starling mechanism was inadequate, since stroke index fell despite the greater initial fiber length. In these dilated hearts, mean systolic force applied to the LV wall increased as the energy of contraction was diverted from fiber shortening to generation of internally directed force. With the increase in afterload caused by greater wall stress, contractility in the limited surviving muscle fell; $\Delta p/\Delta t$ and fiber shortening rate fell and the time needed for development of peak force rose (3, 5). In terms of oxygen cost, "internal contractile element work" used in tension development is more costly than "external contractile element work" used for ejection (39). In hearts with extensive coronary artery disease and local asynergy, the overloaded surviving myocardium may well become relatively hypoxic and shift to a lower function curve.

The degree of asynergy is related to the extent of coronary artery disease, and does not seem to be prevented by the development of collaterals (40). Hemodynamic abnormalities may not become apparent until the heart is stressed, as with exercise or angiotensin (41). In general, marked overall impairment of cardiac function ensues when regional asynergy is so severe and extensive that the uninvolved myocardium cannot compensate adequately despite increased shortening and hypertrophy (42).

The elastic properties of an asynergic area are determined by both its inert components and by the contractile state of its surviving muscle. Of 16 aneurysms examined in Gorlin's series (4), seven showed thin fibrous scars, seven a mixture of fibrous tissue and muscle, and two, surprisingly, seemed composed of normal muscle. Dyskinesis in normal muscle might reflect local disorders in excitation-contraction coupling, in metabolism, or in stores of catecholamines. Acute doses of ethanol increased asynergy in patients with alcoholic cardiomyopathy; the asynergy due to coronary artery disease was decreased by norepinephrine and increased by β-adrenergic blockade (5). Among nine akinetic and eight dyskinetic areas resected and reported in part by Tice (43) there was extensive replacement by scar tissue in all, with only rare and atrophic surviving muscle fibers, and a high incidence of adherent pericardium and mural thrombus. Resting cardiac muscle at normal sarcomere lengths has a compliance of 6–10 percent if it is stretched to the descending limb of the length-tension curve, compliance falls to 2 percent. The resting value is midway between the compliances of collagen, 1 percent, and of elastin, 15 percent; with activation, heart muscle becomes ten times stiffer than at rest (44). These properties help to explain the resistance of normal, active myocardium to systolic expansion and suggest that the proportions and variety of connective tissue and of active or inactive muscle determine the kinetic behavior of a scar.

An experimental model for the effect of a slack SE on contraction was provided by Hill (45) who showed that when springs of increasing compliance were added in series to a contracting muscle the total isometric force and the rate of force development decreased. A recent study placed two papillary muscles in tandem as a model for two connected areas of the ventricular wall (46). Dysynchronous activation affected performance little, but hypoxia in one muscle reduced its force development and permitted it to be stretched "paradoxically" by the normal partner. Experimental myocardial infarction in dogs initially caused no change in overall active stiffness of the heart, but after several months, active stiffness was markedly decreased due to the presence of a thin compliant scar (47). Another study, however, showed an increase in ventricular stiffness at four–five days after acute infarction (48).

SURGICAL RESECTION OF ANEURYSMS

Ventricular aneurysms have been resected with increasing frequency since 1958, and there is convincing evidence that if a large dyskinetic area is removed, the performance of a failing heart can be dramatically improved (49, 50). Akinetic areas are now discovered more often with the increased use of cineangiography to select patients with severe angina for surgery. Resection or exclusion by plication of akinetic scars, some as large as 50 percent of the LV mass, has also produced clinical and hemodynamic improvement (51, 52), and the current problem is to establish indications for operation. Studies based on autopsies have given varying estimates of the effects of aneurysms on life expectancy, but it is clear that long survival is not unusual, especially with small lesions (53). Surgical correction should be considered if a large, localized area of asynergy is associated with chronic congestive heart failure, intractable ventricular arrhythmias, systemic embolism, or if it is contributing to severe dysfunction of an adjacent papillary muscle (54). It is not apparent whether post-infarction aneurysms which are static in size and do not produce symptoms should ever be prophylactically removed. Careful monitoring of the size of the aneurysm and of the size and performance of the heart, as in patients with aortic valvular disease who are asymptomatic, will help to decide when surgery becomes indicated in each patient.

With successful coronary bypass procedures, contractility may improve in the revascularized heart (55), suggesting that if an asynergic area contains enough viable muscle it might regain synergy when the blood supply is improved. Since asynergic areas may contribute to the production of angina, coronary bypass can sometimes be ideally combined with resection of scars.

THE ROLE OF ASYNERGY IN ANGINAL SYNDROME AND ACUTE MYOCARDIAL INFARCTION

Abnormal physical findings during anginal pain or myocardial infarction, such as precordial systolic bulging, gallops and paradoxical splitting of the second sound, suggested that there might be regional asynergy in the ischemic areas, perhaps leading to abnormal ventricular function (2, 56).

Angina.—There is no direct evidence that asynergy occurs acutely and reversibly in otherwise normal myocardium during anginal attacks; no cineangiographic studies during angina have been reported. Transient changes in the hemodynamics of the left ventricle have been observed during angina in man, but their significance is questionable. Increases in pulmonary artery wedge pressure and LV_{EDP} are frequent during spontaneous angina and with angina induced by exercise or rapid atrial pacing. The increase in LV_{EDP}, like S-T segment depression and regional lactate production, usually precedes induced anginal pain (57). In some studies, angina was associated with low systolic ejection rates or with LV stroke work inappropriate to the elevation in LV_{EDP}, suggesting that the ventricle was operating transiently on a depressed function curve (57–60). Other observations showed no changes in cardiac output, mean systolic ejection rate, dp/dt or in left ventricular stroke work, indicating that the ventricle was functioning normally and that the elevation in LV_{EDP} reflected a transient decrease in ventricular compliance during diastole (61–64). As more cineangiographic and hemodynamic studies are made in patients during induced angina, the significance of the changes in ventricular behavior may become better understood.

Myocardial infarction.—If regional ischemia persists beyond 20 min, early changes of myocardial damage become histologically evident as irreversible cell injury occurs and cell death begins (65). Biochemical and functional abnormalities occur much sooner. Ischemia persisting for 20 to 60 sec causes depletion of CrP and absence of contraction, both of which are quickly reversible; at 60 to 200 sec there are probably changes in the function of mitochondrial and reticular membranes, producing changes in oxidative phosphorylation and excitation-contraction coupling which are slowly reversible; after 200 sec, irreversible damage to these membranes occurs (66). The ischemic areas become acidotic, depleted of K^+ and catecholamines, accumulate lipid more rapidly and there is a fall in protein synthesis until resynthesis begins after three–four days (67). The relationship between the size of an infarcted area and the impairment of ventricular function is not clear. Although the size of infarcts found at autopsy was unrelated to the clinical occurrence of shock (68), hearts from patients dying with acute "pump failure" showed a high incidence of acute coronary occlusion and transmural infarction (69). In experimental animals, acute coronary occlusion produces acute depressions in left ventricular function curves, in dp/dt and in force-velocity curves, reflecting the decrease in the number of force-generating sites (70–72). Cardiac output is usually decreased and LV_{EDP} is elevated, but these changes are variable. Left ventricular function recovers after seven days, perhaps as areas adjacent to the infarct become less ischemic and regain catecholamines, as normal muscle hypertrophies and as the infarcted area becomes stiffer (72).

In acute myocardial infarction in man, cardiac output is usually low but can be normal; shock is usually associated with low outputs but equally low values can occur without shock (73–75). Other evidence for left ventricular failure in man includes elevations in pulmonary venous wedge pressure or LV_{EDP} (76–77), and prolongation of the isovolumic contraction period estimated at the bedside (78).

As hemodynamic and biochemical studies increase in coronary care units, more sophisticated assessments of contractility will be made after myocardial infarction. Such measurements may clarify the perplexing situation in which cardiac "pump failure" occurs although only the small infarcted segment is asynergic. This understanding is essential for the proper evaluation of treatment with inotropic and pressor agents, mechanical assistance and, ultimately, regional infarctectomy (79).

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STRUCTURE AND FUNCTION OF THE MYOFIBRILLAR CONTRACTILE PROTEINS[1]

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It is appropriate to begin this review with the central dogma (1, 2) that the myofibril contains an interlocking array of thick filaments (myosin) and thin filaments (actin), and that the primary contractile event involves some kind of linkage between cyclic interaction of thick and thin filaments and enzymatic splitting of adenosine triphosphate (ATP) by myosin, in particular, that part of myosin within cross-bridges that extend outward from the thick filament core (3).

This overall scheme has been elaborated in detail during the past several years, and a number of reviews has treated special areas of research, including the X-ray analysis of myofibrillar structure (4, 5), the structure and function of myosin (6, 7), the role of Ca^{++} in contraction (8) and characterization of the tropomyosin complex (9), and the energetics of contraction (10, 11). Among many excellent reviews, we would draw attention to the comprehensive review by Katz (12) on the contractile proteins of the heart, to the highly informative (and delightful reading) review by Sandow (13) on skeletal muscle, and to the published proceedings of recent symposia on the mechanism of muscular contraction (14–19). The present review is limited in space and will focus on those aspects that seem to us to be among the most important, especially with respect to potential impact on clinical medicine.

MYOSIN

Electron microscopic studies.—The myosin molecule is large enough to visualize on electron microscopy and appears to have a rodlike tail approximately 1300 Å in length and 20 Å in diameter, that terminates in a globular head roughly 200 Å in length and 50 Å in diameter (20–23). More recent studies (24, 25) show the globular head of myosin to have a bilobed structure in a large proportion of the molecules visualized.

The myosin molecule may be cleaved by trypsin into two fragments, light meromyosin and heavy meromyosin (26–28). Light meromyosin forms the thick filament core (22), whereas heavy meromyosin retains the characteristic ATPase and actin-binding properties of myosin (26, 27) and forms the cross-bridges (22). On electron microscopy, light meromyosin comprises about two-thirds of the rodlike tail of myosin and heavy meromyosin comprises the rest of the rodlike portion and the globular head of myosin (21–23, 29).

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Heavy meromyosin can be digested further with trypsin (30) or papain (31, 32) into a smaller fragment, termed subfragment-1 (30), that retains ATPase and actin-binding properties. Subfragment-1 appears as a globular fragment on electron microscopy (21, 24, 25), and it has been concluded that each particle of subfragment-1 may be identified with one lobe of the globular head of myosin (24, 25). Although some caution must be expressed in the detailed interpretation of protein fragments from their appearance on electron microscopy, there is considerable chemical evidence (described below) that each myosin molecule does contain two particles of subfragment-1.

Subunit composition.—Most investigators (33–37) have reported weight-average molecular weight values about 510,000 for rabbit skeletal myosin prepared by the customary Szent-Györgyi method (38); however, molecular weight values of 600,000 were described (39–42) for myosin prepared by ammonium sulfate fractionation. The discrepancy between these results has been attributed to aggregation of myosin during ammonium sulfate precipitation (35, 43, 44), as shown, for example, by sedimentation velocity experiments on ammonium sulfate-treated myosin (45). But one still questions whether aggregation has contributed to the 510,000 value for myosin and, indeed, a progressive increase in weight-average molecular weight was noted in light-scattering experiments on stored myosin (47) and during prolonged ultracentrifugation of myosin (45, 48). Detailed ultracentrifugal studies involving high-speed sedimentation equilibrium experiments (45, 46, 48), Archibald measurements (46, 48), and conventional sedimentation equilibrium experiments (46, 48) indicate that myosin preparations contain a predominant monomer fraction of 468,000 mol wt and aggregated protein, the proportion of which increases with time and varies with the method of preparation. Recently, Godfrey & Harrington (49, 50) have asserted that the relatively high weight-average molecular weight values earlier reported (39, 40) from their laboratory reflect a reversible monomer-dimer equilibrium for myosin; however, this interpretation is not consistent with the evidence for time-dependent aggregation of myosin (46–48), nor is it supported by any direct evidence of time-stability during prolonged ultracentrifugation. Furthermore, gel diffusion experiments have been consistent with irreversible aggregation of myosin without evidence for a reversible monomer-dimer equilibrium (see Reference 303 for recent work using Sepharose 48). One might also note that the principal evidence favoring the alleged monomer-dimer equilibrium, namely, a slight decrease of apparent molecular weight at low radial distances and low protein concentrations in sedimentation equilibrium experiments on myosin (49), may alternatively be attributed to the partial dissociation of light chains from myosin (see below) or simply the presence of low molecular weight contaminants in the preparation; unfortunately, neither possibility was explored in these studies (49, 50). Accordingly, we believe there is no compelling reason to question the prevailing interpretation that myosin exists predominantly as monomer in solution at high ionic strength, and that aggregation of myosin reflects some time-dependent denaturation process, the exact nature of which remains as yet unknown.

It was early shown that myosin liberates light and heavy components after prolonged treatment in 6.7 *M* urea (51), and Kominz et al (52) described release from alkaline-treated myosin of a low molecular weight component with well-defined amino acid content and C-terminal isoleucine. Others have confirmed that low molecular weight material is released from myosin in urea solutions (53, 54), at pH 11 (55), and on heat treatment (56, 57); but it was widely believed that this material represented contaminant proteins or proteolytic fragments of myosin (e.g., 58, 59). This interpretation was based largely on the physicochemical studies of Kielley, Harrington, and associates (39, 40, 60, 61), that were taken to imply dissociation of myosin by 5 *M* guanidine into three equivalent subunits. However, this analysis (39, 40, 60, 61) has been criticized in several crucial respects (see 44, 45); and Dreizen, Hartshorne & Stracher (62) have demonstrated quite unambiguously that purified preparations of myosin are, in fact, heterogeneous in 5 *M* guanidine.

The nature of this heterogeneity was characterized by multicomponent analysis of high-speed sedimentation equilibrium experiments, which indicate dissociation of myosin by 5 *M* guanidine into a heavy component (~ 215,000 mol wt) and a light component (~ 20,000 mol wt) that comprises 10 to 15 percent of the total protein (45, 46, 63). Ultracentrifugal studies on the purified heavy chains confirm a molecular weight of 212,000 (± 5000) and indicate their essential weight-equivalence (48). Other work on purified heavy chains indicates molecular weight values of roughly 235,000 from viscosity (64) and 194,000 from high-speed sedimentation equilibrium (65); however, the latter workers (65) fail to identify a minor contribution of residual light component in their analysis, with consequent underestimation of heavy chain weight by 5–10 percent, and seem to confuse the molecular weight of heavy chains with the weight-average molecular weight (~ 195,000) of dissociated myosin, as determined earlier in their laboratory (40) and calculated from the subunit composition of myosin (45, 48, 63).

More precise characterization of the myosin light component was achieved from ultracentrifugal studies on myosin at pH 11 (46, 48, 63), which indicate the presence of 12 percent light component, with molecular weight values of 20,200 for light component and 420,000 for heavy component. Assuming 470,000 molecular weight for myosin, these data indicate alkaline dissociation of nearly three light chains from the intact heavy chain core (46, 48, 63), and this result is supported (less exactly) by the difference in molecular weight between intact myosin and heavy alkali component and other evidence that approximately 3.5 C-terminal isoleucine residues of native myosin belong to the light chains (48, 63, 66).

Comparable stoichiometry and molecular weight for the light chains of myosin were described independently by Locker & Hagyard (67) from experiments based on dissociation of myosin by acetylation. Although Frederiksen & Holtzer (64) reported a molecular weight above 30,000 for the purified light chains, this result may reflect the propensity of the light chains to undergo aggregation (7, 48). Less than 12 percent light component in myosin has also been reported, but one such study (68) seems to be on a small fraction of the total light component, and the other (65) depends on the use of an extinction coefficient that is uniquely high and also inconsistent with known amino acid content of the light chains (52, 65, 67, 68). Chromatographic studies indicate that the proportion of light component in myosin is not significantly changed on single passage through DEAE-cellulose (48, 67, 69), DEAE-Sephadex (70, 71), or cellulose phosphate (48).

The light chains may be fully dissociated from the intact heavy chain core of myosin on heat treatment (48, 72), in 2 *M* guanidine (48), and in concentrated salt solutions at neutral pH (46, 72–74); and at least some light component may be dissociated from myosin by mercurial reagents, like methylmercuric hydroxide (75) and *p*-chlormercuriphenylsulfonate (76). As noted below, use of these different dissociating methods turns out to be most helpful in relating structure to function in myosin.

There are recent attempts to determine the molecular weight of light chains from their properties on acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Weber & Osborn (304) report three bands of 17,000 to 23,000 molecular weight for the light component of myosin, consistent with the 20,000 weight-average value from ultracentrifugal experiments. However, Paterson & Strohman (305) present data showing electrophoretic bands of 19,000 mol wt, 33,000 mol wt, and still higher molecular weight; and these results have been interpreted in terms of discrete polypeptide chains (305). In evaluating this discrepancy, we would note that comparable high molecular weight heterogeneity has been reported (7, 48, 74) in ultracentrifugal experiments on myosin light component and attributed to aggregation of light chains, since the extent of this heterogeneity varies with specific denaturing conditions and is negligible on dissociation of light chains from carboxymethylated myosin (63) and acetylated myosin (67).

The light and heavy chains of myosin are held together by noncovalent forces (62–64), without evidence of disulfide bridges as found, for example, between the light and heavy chains of the immunoglobulins. Analysis of heat denaturation data on myosin indicates a free energy about 10 to 15 kcal/mole for the interaction between light and heavy chains (7). The interaction zone between light and heavy chains involves predominantly polar groups, rather than hydrophobic groups (7, 72); and the dissociation of light chains from myosin at pH 11 is accompanied by the ionization of about four to five basic residues per light chain (7).

Subunit heterogeneity.—Myosin contains approximately 36 sulfhydryl groups, and there are 15 (77, 78) or 16 (79, 80) different thiol peptides consistently and an additional 6 to 9 thiol peptides irregularly (77–80). These data suggest an essential twofold symmetry of the myosin molecule, with some indication of microheterogeneity of the light or heavy chains. Despite an early report that the light chains of myosin show similarity in their peptide maps and identical thiol sequences (71), futher work indicates that the light chains contain at least two different thiol sequences (81).

The light chains are electrophoretically heterogeneous, with three or four major bands for the light component dissociated from carboxymethylated myosin at pH 11 (63) and from acetylated myosin (67, 82). Comparable electrophoretic patterns after dissociation of light chains from chemically unmodified myosin at pH 11, in 2 *M* guanidine, and in concentrated salt solutions indicates that electrophoretic heterogeneity derives, at least in part, from true structural differences among the light chains (74, 83). Nevertheless, prolonged alkaline and salt treatment does result in modification of the electrophoretic pattern (74, 83, 84) and aggregation of light chains (7, 48, 74); and irreversible denaturation presumably accounts for the extensive heterogeneity of the light alkali component reported by some (65, 68, 76).

In a significant observation, Locker & Hagyard (85, 86) have described different electrophoretic patterns for light component dissociated from myosin of red, white, and cardiac muscle by acetylation; and electrophoretic differences between the light chains of red and white skeletal myosin have been confirmed following alkaline dissociation (84, 87, 88) and reaction with *p*-chlormercuriphenylsulfonate (76). The specific electrophoretic patterns vary widely and the reason for these differences is uncertain at present.

A fraction of the total light component which is dissociated from myosin by NH_4Cl treatment (74, 83) contains predominantly fast band on cellulose acetate electrophoresis (73, 74). This protein comprises approximately one mole per mole of myosin, is found in preparations of red and white skeletal myosin (84, 88) as well as cardiac myosin (89), is not essential for the Ca-ATPase activity of myosin (see below), and appears to contain a low-affinity site for Ca^{++} (90).

Differences between the heavy chains of red and white skeletal myosin are shown by the greater susceptibility of white skeletal myosin to tryptic digestion (91–95) and the absence of 3-methyl histidine in red skeletal myosin (96). Gel electrophoretic experiments show one band (60) or multiple bands (97) for the heavy chains, but interpretation seems ambiguous in view of possible contribution for heavy chain aggregates.

There is also evidence of significant differences between fetal and adult skeletal myosin with respect to sedimentation properties (36, 98), heavy chain content of 3-methyl histidine (96, 99) and other amino acids (100), and electrophoretic patterns of the light chains (101).

Structure of myosin.—Lowey & Cohen (102) concluded that light meromyosin has a double-stranded α-helical conformation, based on comparison of amino acid content and hydrodynamic estimates of length. Although these calculations have been disputed (103, 104), ultracentrifugal studies (7, 46, 63) indicate that light meromyosin remains intact at pH 11, without release of any light chains; and osmotic pressure data (105) are consistent with the dissociation of light meromyosin by 8 *M* urea into two fragments. Heavy meromyosin has a more complex structure and is dissociated at pH 11 into a remnant of the heavy chain core (~ 250,000 mol wt) and most if not all of the light component of intact myosin (7, 46, 63). The light component liberated on acetylation (67) or succinylation (106) is also found in heavy meromyosin, as are the C-terminal isoleucine residues of the light chains (63, 66).

Subfragment-1 comprises 70 to 80 percent of heavy meromyosin (107, 108) and has molecular weight about 110,000 (107, 109–112); this stoichiometry would suggest that heavy meromyosin yields two particles of subfragment-1 (44, 108). Ultracentrifugal experiments (112) indicate that papain-prepared subfragment-1 is dissociated by 5 *M* guanidine and at pH 11 into remnants of one heavy chain, 85,000 in weight, and one light chain, 18,000 in weight. Trypsin-prepared subfragment-1 is a more degraded particle, containing a remnant of one heavy chain (65,000 molecular weight), one light chain (18,000 molecular weight), and considerable peptide material (112). That each myosin molecule contains two particles of subfragment-1 is supported by stoichiometric evidence on 3-methyl histidine (113) and other trace amino acids (100), reactive thiol groups (112, 114, 115), pyrophosphate binding sites (108, 116), and fluorodinitrobenzene binding sites (117) in myosin and subfragment-1.

The overall findings on the structure of myosin are summarized in Figure 1 and follow the original proposal of Gershman, Dreizen & Stracher (63) that native myosin comprises an axial core of two heavy polypeptide chains (210,000 mol wt) that terminate in a globular head containing three polypeptide chains (20,200 average mol wt). Subfragment-1 contains remnants of one heavy chain and one light chain (112), and recombination experiments on ATPase activity and nucleotide binding (described below) would support the conclusion that myosin contains two enzymatically active protomers, each comprising one heavy chain and one light chain. The present evidence (73, 74, 83, 118–120) would indicate that the two essential light chains are sampled from a population of three or more electrophoreti-

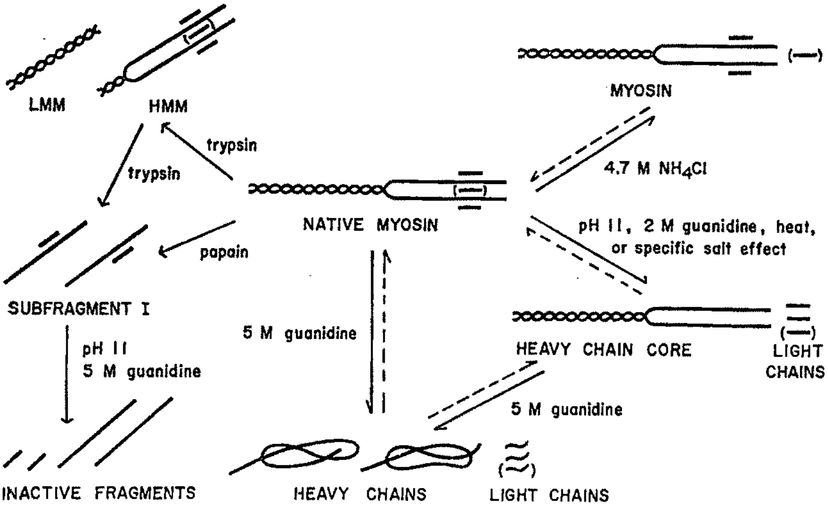

FIGURE 1. Subunit structure and interactions of myosin (from 7).

cally different components; the nonessential light chain comprises a single (fast) electrophoretic component that may be considered tentatively a distinct protein, termed "myonin" (7), closely associated with myosin proper.

Relationship of ATPase to subunit interactions.—In general, those denaturing conditions which result in dissociation of light chains from myosin also lead to irreversible inactivation of ATPase. Thus, on storage of myosin at temperature above 25°C, there is loss of ATPase and light chain dissociation, with comparable values of activation enthalpy (55 kcal/mole) and activation entropy (100 eu) for both processes (48, 72). Similarly, myosin ATPase is irreversibly denatured on alkaline treatment above pH 10 (45, 48, 63, 68, 121–123), over the same range of pH where light chains are dissociated from myosin (45, 48, 63). The loss of ATPase precedes light chain dissociation (48) and is presumably due to alkaline hydrolysis of critical residues at or near the ATPase site.

A close relationship between dissociation of light chains from myosin and rapid irreversible loss of ATPase has also been found in a series of experiments on myosin in concentrated salt solutions (46, 72, 118, 120). Treatment of myosin in those salt solutions which fully dissociate light chains from the heavy chain core is accompanied by rapid irreversible loss of ATPase at or slightly below the transition concentration for light chain dissociation. However, some of the light component is not essential for myosin ATPase, as shown by nearly full recovery of Ca-ATPase despite irreversible dissociation of one mole light chain per mole myosin (73, 74, 83, 120). Some light component is also dissociated from myosin on treatment with *p*-chlormercuriphenylsulfonate (76) and DTNB-urea (65) without net loss of ATPase activity, but these results seem ambiguous owing to possible activation of Ca-ATPase by sulfhydryl reagents.

More direct evidence of a dependence of ATPase on subunit interactions has been sought in recombination experiments on myosin. Frederiksen & Holtzer (64) reported that light and heavy alkali components alone showed no ATPase activity, while myosin titrated to pH 11 and back-titrated to pH 7 retained 50–80 percent of ATPase, the extent of activity independent of the duration of alkaline treatment; however, these results are disparate with well-documented evidence of time-dependent alkali denaturation of myosin ATPase (48, 68, 121–123). In other work, Gershman, Stracher & Dreizen (48) reported that 10 to 15 percent ATPase is recovered after dissociation and reconstitution of myosin in 2 *M* KCl-0.01 *M* ATP at pH 11, while protein reconstituted with less than full complement of light component shows significantly less ATPase activity. In experiments involving dissociation of myosin in 4 *M* LiCl and subunit fractionation by citrate salting out (118–120), fully reconstituted myosin retains about 70 percent of control ATPase (provided that thiol groups are protected); but protein with less than 8 percent light component shows a sharp fall in ATPase, and light chains alone exhibit no activity. Following chromatographic fractionation of myosin in 4 *M* LiCl (124), about 30 percent ATPase is recovered in reconstituted myosin, and again no activity in purified light and heavy components. Finally, recombination experiments involving LiCl-citrate fractionation of NH_4Cl-treated myosin (two heavy chains and two light chains), confirm that recovery of Ca-ATPase requires a molar ratio of essential light to heavy chains (119, 120).

The recombination experiments indicate that some kind of interaction between essential light and heavy chains is involved in the ATPase activity of myosin (48, 118–120, 124). One might suppose that residues from essential light and heavy chains participate at or near the hydrolytic site, or, alternatively, that one subunit, say the heavy chain, may contain the hydrolytic site and the adjacent subunit (essential light chain, according to this model) may stabilize the unique conformation required for enzymatic activity.

The ATPase activity of white myosin exceeds that of red muscle myosin (86, 92, 95, 125–127), and there are significant differences in the light and heavy chains of red and white skeletal myosin (see above). Hybridization experiments on red and white skeletal myosin indicate that the level of ATPase depends on light chain and probably also heavy chain composition 88, 88a). Fetal myosin exhibits less ATPase activity than adult myosin (36), and this difference, too, is presumably related to specific subunit composition (101). The overall evidence would strongly support the hypothesis, originally suggested by Trayer & Perry (36), that myosin be considered an isoenzyme system.

Nucleotide binding.—Myosin and heavy meromyosin contain 1.2 to 1.8

binding sites for ATP (128, 129), ADP (130, 131), and pyrophosphate (116, 132). As subfragment-1 contains only one binding site for ADP (133) or pyrophosphate (116), most workers (116, 129–132) have concluded that myosin contains two equivalent, independent binding sites for nucleotide. Actually, myosin (or heavy meromyosin) contains less than twice the number of nucleotide binding sites of subfragment-1, and it has been noted (7) that this effect would be indicative of negative cooperativity between binding sites on symmetric protomers of myosin, in which nucleotide binding to one site inhibits nucleotide binding to the other site.

Following LiCl-citrate fractionation of myosin, recombination experiments indicate 1.6 ADP binding sites per molecule of reconstituted myosin, with or without the NH_4Cl dissociated light chain present (7, 119, 120). The number of ADP binding sites is diminished in protein containing fewer light chains, and heavy chain core and light chains alone do not exhibit significant binding. This would suggest that nucleotide binding involves some kind of interaction between light and heavy chains. The simplest explanation would be that nucleotide bridges essential light and heavy chains within each protomer, but this interpretation does not exclude the possibility of weak interaction of nucleotide with light or heavy chains alone. That this may be the case is suggested by evidence (134) that an analog of ATP is bound covalently to a fraction of the total light alkali component.

The subunit structure of myosin is stabilized by ATP to alkaline (48, 118) and salt (118, 119) dissociation, and this effect has been related to interaction of myosin with the terminal phosphate groups of ATP (7, 84). Electrophoretic experiments show further that stabilization involves only the two essential light chains (135). These findings taken in conjunction with the recombination experiments on ATPase and ADP binding suggest that the triphosphate part of ATP directly bridges essential light and heavy chains in the region of the hydrolytic site (7, 84, 119, 135). Alternatively, one might hypothesize (7, 84) an allosteric relationship between nucleotide binding and stabilization of subunit interactions, but this explanation would not be consistent with the lack of catalytic activity in light or heavy chains alone.

Several sulfhydryl groups have been implicated in the hydrolysis of ATP by myosin. Reaction of one of these groups (S_1) with N-ethylmaleimide activates Ca-ATPase (136), whereas reaction of the other group (S_2) inactivates Ca-ATPase (137). Both S_1 and S_2 groups are located on the heavy chains of myosin (45, 112, 115); and peptide fragments containing a high proportion of S_1 and S_2 groups may be extracted by tryptic digestion of myosin without significant effect on ATPase (112, 114, 115), suggesting that S_1 and S_2 groups have a regulatory role in ATP hydrolysis. Sekine & Kielley (138) have shown that the association between substrate and active site is greatly modified in S_1-activated myosin. Recent studies (7, 119, 135) indicate a linkage between sulfhydryl reactivity and subunit interactions, as shown by diminished stability of S_1-reacted myosin than native myosin to

LiCl denaturation. Nucleotides and chain phosphates do not modify the LiCl denaturation profile of S_1-reacted myosin (7, 119, 135), suggesting that S_1-reacted myosin has undergone a change in quarternary structure involving the contact zone between light and heavy chains at or near bound nucleotide.

Reaction of myosin with ATP is not accompanied by any drastic change in the overall structure of myosin, at least as evident from sedimentation and optical rotatory dispersion measurements (306). However, reaction of myosin with fluorescent labels (307, 308) and spin-labels (309, 310) does demonstrate minor conformational changes at or near the active site in response to thiol reaction and pH change. One may anticipate considerably more work involving perturbation of the myosin-active site by covalent reaction, especially in conjunction with peptide analysis, as this kind of approach offers one of the best means now available (pending X-ray analysis of myosin) for exploring the structural changes that accompany reaction of myosin with nucleotide.

Mechanism of ATPase.—Enzymatic splitting of ATP by myosin involves hydrolysis of the bond between the terminal phosphorus and its bridge oxygen atom (139), and exchange studies of O^{18} incorporation into inorganic phosphate (P_i) are suggestive of transient phosphorylation of myosin (140, 141). As efforts to isolate a phosphoryl intermediate were unsuccessful, Sartorelli et al (142) suggested alternatively that ATP hydrolysis is followed by electrostatic binding of metaphosphate to myosin and subsequent hydration to P_i.

Extensive studies have been reported by Tonomura and co-workers (143–146) on the initial burst of P_i liberation that precedes steady-state hydrolysis of ATP. The extent of initial burst varies with solvent composition, reaching a maximum slightly greater than 1 mole P_i per mole myosin (143, 145). The initial burst of P_i and concomitant proton release (144) have been attributed to phosphorylation of myosin (143–145), and a complex reaction mechanism has been proposed (146). More recently, Taylor and co-workers (147–149) confirm the initial burst of P_i and protons, but describe different rate constants and arrive at a simpler kinetic scheme that involves (a) binding of ATP, (b) hydrolysis of ATP, and (c) dissociation of ADP and P_i from myosin, with the rate-limiting step being product dissociation. The extent of P_i release extrapolates to 1.7 mole per mole of myosin, with indication of more than one class of sites (148). Preliminary evidence was presented that the two sites of myosin interact with one another (147, 149), and one looks forward to kinetic measurements on subfragment-1 for resolution of this important question.

ACTIN AND THE INTERACTION OF ACTIN WITH MYOSIN

Actin.—The major protein of the thin filament, actin, may be isolated as a monomer (G-actin) that undergoes polymerization to F-actin (150) under appropriate conditions. Most early work on actin was complicated by the presence of tropomyosin and other contaminant proteins, and/or aggregation of purified actin, especially without thiol protection (151, 152). Sedimentation equilibrium experiments done with care to protect thiol groups indicate a molecular weight of 47,000 for G-actin (151, 153), and this result is consistent with the presence of one mole of 3-methyl histidine (113), one mole of bound ATP (153, 154), and one mole of bound Ca^{++} (155) per 45,000 to 50,000 g of actin.

Actin has a globular appearance on electron microscopy (156). As hydrodynamic measurements (157, 158) favor a somewhat more elongated structure (axial ratio 2–3), it has been suggested (157) that actin contains a flexible region near the nucleotide binding site. That actin comprises a single polypeptide chain is indicated by ultracentrifugal experiments in 5 *M* guanidine (152, 153), a single C-terminal group in molar ratio (152, 159), and the absence of symmetry of thiol peptides (160) or cyanogen bromide peptides (161, 162).

At least two laboratories (161, 162) are working actively on the primary structure of actin. Published reports indicate cleavage of actin by cyanogen bromide into 17 peptides (161), seven of which have been sequenced (162), and we may anticipate determination of the complete amino acid sequence of rabbit skeletal actin within the next year or so.

It was early shown (163, 164) and confirmed repeatedly that native G-actin binds ATP in molar ratio, and that polymerization of G-actin to F-actin is accompanied by splitting of P_i from bound nucleotide. While ATP bound to G-actin exchanges rapidly with solvent ligands, nucleotide bound to F-actin is not readily exchangeable (165); the extent of exchangeability is increased by sonication (166, 167), and decreased in the presence of tropomyosin (168, 169) or myosin (169). The G-to-F transformation ordinarily requires the presence of bound nucleotide, but can proceed in its absence in 50 percent sucrose (170). These findings suggest that nucleotide stabilizes the actin molecule during polymerization, but is not involved in direct bridging of actin monomers (169, 170). The function of bound nucleotide in the interaction of actin with myosin has been controversial (171), and it has been asserted (167) that bound ADP does not affect activation of myosin ATPase by actin or the superprecipitation reaction. Nevertheless, actin-bound nucleotide would affect surface charge of the thin filament and in this way, if not otherwise, modify the interaction of thick and thin filaments.

Oosawa and associates (172, 173) have shown that the G-to-F transformation is not a simple end-to-end linear polymerization but a complex reaction that involves nucleation and growth processes and proceeds via cooperative interactions to a unique helical structure. The actin-actin interaction appears to involve a single tyrosine residue per actin molecule (174), implying that actin is asymmetric with respect to polymerization. Evidence has been presented (169, 175, 176) that cyclic dephosphorylation of ATP by F-actin is accompanied by interruption of the helical region lying adjacent to the nucleotide and it has been suggested that such changes may play an important role in the contractile process.

Actin-myosin binding.—In solution at 0.6 *M*, actomyosin contains myosin and F-actin in 3.7/1 weight ratio (177–179), indicating binding of 1 myosin molecule per 2.7 actin monomers. However, at low ionic strength, heavy meromyosin appears to bind actin in molar ratio (180, 181). As each actin monomer binds one particle of subfragment-1 under similar solvent conditions, Young (180) has suggested that only one of the globular heads of heavy meromyosin interacts with a single actin molecule at a time. Recent electron microscopic experiments by Moore, Huxley & DeRosier (182) show directly that F-actin can be decorated with subfragment-1 in close to one-to-one ratio. Although the acto-subfragment-1 interaction site has a unique configuration (182), it remains somewhat uncertain whether this arrangement derives from close packing of subfragment-1 particles or the specific interaction between actin and subfragment-1.

The studies on actin-myosin binding are given added significance by evidence (183) that the kinetic parameters for inhibition of K^+-ATPase and activation of Mg^{++}-ATPase of heavy meromyosin by actin reflect the interaction of heavy meromyosin and actin in molar ratio, and Rizzino et al (183) surmise that binding of actin to one head of heavy meromyosin may influence the enzymatic site on the other head of heavy meromyosin.

Actomyosin ATPase.—In general, myosin ATPase is inhibited by Mg^{++}, whereas at low ionic strength actomyosin ATPase is activated by Mg^{++} (184). At high ionic strength, actomyosin is dissociated into actin and myosin, and ATPase is inhibited. This would suggest that Mg^{++} activation of ATPase is characteristic of undissociated actomyosin. However, taking advantage of the fact that heavy meromyosin, unlike myosin, does not undergo association into filaments at low ionic strength, Perry and co-workers (185–187) studied the acto-heavy meromyosin system at low ionic strength and found, interestingly, that Mg^{++} activation of ATPase is accompanied by dissociation of acto-heavy meromyosin into F-actin and heavy meromyosin. Thus, despite disruption of the tight actin-myosin linkage by Mg^{++}-ATP, there is residual interaction of actin with myosin, presumably some kind of cyclic effect that leads to transient conformational change at the hydrolytic site (185). This interpretation is supported by evidence that in the absence of Mg^{++}, addition of actin leads to inhibition of myosin ATPase (188).

In a detailed kinetic study of the effect of actin on heavy meromyosin ATPase (189–192), Eisenberg & Moos have shown that fully complexed acto-heavy meromyosin (at infinite ratio of actin to heavy meromyosin) exhibits ATPase about ten times that of ordinary acto-heavy meromyosin, suggesting that ordinary acto-heavy meromyosin is, in reality, largely dissociated into F-actin and heavy meromyosin This would imply that the true

ATPase of actomyosin in vitro approaches the ATPase rate of maximally working skeletal muscle (193). Change in ionic strength affects the binding of actin to heavy meromyosin without affecting ATPase activity of the acto-heavy meromyosin complex (191, 192), and actin and ATP can bind simultaneously to heavy meromyosin, although Mg^{++}-ATP greatly enhances the dissociation of acto-heavy meromyosin. Despite early reports (194, 195) that subfragment-1 ATPase is activated slightly, if at all, by actin and Mg^{++}; recent studies (196, 197) indicate that subfragment-1 ATPase, like heavy meromyosin ATPase, is activated by actin in the presence of Mg^{++}. The kinetic data, according to Eisenberg, Zobel & Moos (196), would support a scheme for muscular contraction in which repeated cycles of actin-myosin linkage in conjunction with ATP hydrolysis is followed by dissociation of actin and myosin and rebinding of ATP at the hydrolytic site.

Other workers (132, 198, 199) have suggested instead that ATP acts both at a regulatory site and a hydrolytic site on myosin. For example, Kominz (199) proposed a scheme involving cyclic association of actin and myosin and ATP hydrolysis, in which ATP is bound successively at clearing site and hydrolytic site. It seems to us that the question of one or two distinct ATP binding sites is greatly complicated by the question of negative cooperativity between the myosin protomers. The experiments so far done on subfragment-1 have not been decisive in this respect, largely because turbidity and viscosity measurements on the acto-subfragment-1 interaction are not as informative as comparable measurements on actomyosin. A way out of this difficulty would involve kinetic measurements on "one-headed" myosin molecules, that is, those in which one of the two heads of myosin is selectively split off by papain (25); but these experiments have not yet been done.

In studies on actomyosin ATPase, Kanazawa & Tonomura (200) describe an initial burst of P_i over two moles per mole of myosin, significantly greater than the initial burst determined for myosin alone under similar solvent conditions, and this effect was correlated with the dissociation of actomyosin (201). Addition of actin to myosin-ATP solutions leads to increase in steady-state release of protons and P_i (202) without evidence of initial burst (203), and it was suggested that actin inhibits the formation of phosphoryl myosin but enhances the dissociation of ADP and inorganic phosphate from myosin; however, there is no direct evidence of a phosphoryl intermediate for actomyosin.

THE TROPOMYOSIN-TROPONIN COMPLEX AND THE REGULATION OF ACTOMYOSIN ATPASE

Tropomyosin.—The history of tropomyosin has all the aspects of a good detective story, the end of which is almost but not quite yet in sight. Tropomyosin was isolated as a distinct myofibrillar protein by Bailey (204) only several years after the discovery of actin (150). Yet, although tropomyosin represents a major fraction of the total myofibrillar protein (Table 1), until

TABLE 1. STRUCTURAL PROPERTIES OF THE MYOFIBRILLAR CONTRACTILE PROTEINS[a]

	Myosin	Actin	Tropomyosin	Troponin
Location	thick filament	thin filament	thin filament	thin filament
Percent total protein	50–55%	20–25%	4–5%	5–7%
Molecular weight	470,000	47,000	70,000	90,000
$S^{\circ}_{20,w}$	6.1S	3.0S	2.7S	3.8S
Shape	bilobed head (200Å) rodlike tail (1,300Å)	globular (55Å)	rodlike (400Å)	globular
Helical content	head 33% tail 94%		90%	42%
Subunits (mol wt)	2 heavy chains (210,000) 3 light chains (20,000) 2 essential[b] 1 nonessential	1 chain	2 chains (35,000)	3 chains A: 20,000 B: 26,000 42,000

[a] Compiled from references cited in text, mostly on rabbit skeletal proteins.

[b] Electrophoretically heterogeneous.

recently its role in contraction and its very location in the myofibril remained unknown.

Early studies (204, 205) indicated tropomyosin to have solubility properties and amino acid composition similar to those of myosin, but a molecular weight as low as 50,000 (206) or 90,000 (205). Tropomyosin was crystallized into elongate fibers with α-pattern on X-ray diffraction (207), and comparison of hydrodynamic measurements with crystallographic data were considered indicative of a two-chain structure (207) close to 400 Å in length (206).

Rather surprisingly, in comparison with work on other contractile proteins, these conclusions have withstood subsequent analysis. A molecular weight value of 70,000 was determined by light scattering (208), several ultracentrifugal methods (209–211) and osmotic pressure measurements (210). Tropomyosin is dissociated by 8 *M* urea into two weight-equivalent subunits, as shown by sedimentation equilibrium (209, 211, 212) and other measurements (210, 213). Although some evidence has been reported for disulfide bridge(s) between the two chains (208, 213), other studies (209–212) indicate that the tropomyosin structure is stabilized by noncovalent interactions, at least in native state. The tropomyosin subunits yield a single band on gel electrophoresis in urea (209, 211), but there remains some question of heterogeneity (or contamination with troponin), in view of reports of two C-terminal groups (214), two N-terminal groups (215), and two discrete electrophoretic bands (216).

On electron microscopy, the tropomyosin molecule has a rodlike appearance, 400 Å in length and 20–30 Å in width (217, 218). The X-ray diffraction pattern (207, 219) and optical rotatory dispersion (210, 220) of tropomyosin are consistent with a coiled-coil α-helical conformation, with some evidence for a structurally loose region (221, 222).

Tropomyosin crystallizes in an orthothombic lattice of unit cell about 400 Å (22, 223, 224), and the similarity of this structure to the Z-line suggested that tropomyosin might be located at least in part within the Z-line (22). Others, noting the presence of tropomyosin in preparations of actin (225) and the stoichiometric interaction of tropomyosin with actin (226–228), suggested that tropomyosin and actin might be associated in situ (156, 226–228); and this interpretation has been substantiated by subsequent work. Thus, the thin filament has a 400 Å periodicity on electron microscopy (156, 229) and X-ray diffraction (230) that is not attributable to actin but corresponds with the period for crystals of tropomyosin (22, 223, 224). More directly, fluorescent-labeled antitropomyosin stains the thin filament throughout (231, 232), while the Z-line is not stained (231) or stained intermittently (232). Finally, evidence that actomyosin ATPase is markedly affected by tropomyosin (233, 234) suggests a regulatory role for tropomyosin in the contractile process (9, 12).

Troponin.—In the course of studies on the EGTA-sensitizing factor (see below), it became evident to Ebashi & Kodama (235) that native tropomyosin contains an additional protein, termed "troponin" (236), which promotes the association of tropomyosin into aggregates. Unlike tropomyosin, troponin does not bind strongly to actin, yet the troponin-tropomyosin complex enhances the polymerization of F-actin (236). Electron microscopic pictures show that ferritin-labeled antitroponin is situated at 400 Å intervals along the thin filament (237), and that troponin is found at 400 Å periods along the tropomyosin lattice in cocrystals of troponin and tropomyosin (224). These findings would indicate that troponin is in close juxtaposition with tropomyosin within the thin filament (236), either at a unique binding site of tropomyosin or between tropomyosin molecules. Significantly, the Ca^{++}-binding capacity of native tropomyosin is contained almost entirely within the troponin fraction (238–240), with estimates of two (239) or four (238, 240) Ca^{++}-binding sites per 100,000 g of troponin.

Studies on the structure of troponin indicate a globular protein with axial ratio about 6 based on intrinsic viscosity (235), a molecular weight estimated variously as 80,000 from sedimentation (9) and 50,000 from light scattering (240), and approximately 42 percent helical content from optical rotatory dispersion (241). Troponin has a multichain structure, as shown by Hartshorne and colleagues (241, 242), comprising two major components, troponin A ($\sim$15 percent) and troponin B ($\sim$65 percent). Troponin A comprises a single polypeptide chain of 20,000 molecular weight (243), as indicated by sedimentation equilibrium experiments in 1 *M* KCl (243, 244) and 5 M guanidine (243). Troponin A is highly negatively charged (245) and contains the high-affinity Ca^{++} sites of troponin (242, 245). Troponin B has a molecular weight of 70,000 on high-speed sedimentation equilibrium (243) and is dissociated by 0.1 *M* HCl (241) and 2 *M* guanidine (243) into two subunits, with molecular weight values about 26,000 and 42,000 from high-speed sedimentation equilibrium and gel electrophoresis. The troponin molecule contains A and B components in molar ratio (241, 242) and has a molecular weight of approximately 90,000 from the recent studies (243).

From preliminary estimates of 5 percent tropomyosin and 3 percent troponin in total myofibrillar protein, Ebashi & Endo (9) suggested the presence of two moles of tropomyosin per mole of troponin. However, Hartshorne & Mueller reported a weight ratio of 1/1.3 for tropomyosin to troponin, based on extraction of these proteins from natural actomyosin (246) and optimal EGTA-sensitizing effect for actomyosin ATPase (247), and this ratio was confirmed in experiments involving superprecipitation of actomyosin (248). This stoichiometry (246–248) would imply that troponin and tropomyosin are found in molar ratio along the thin filament, and the discrepancy with the earlier result (9) may possibly reflect the presence of additional tropomyosin within the Z-band.

Regulation of actomyosin ATPase: the EGTA-sensitizing factor.—There is now considerable evidence for the primary role of Ca^{++} in regulating muscular contraction (see 8, 9), namely, that excitation initiates movement of Ca^{++} from sarcoplasmic reticulum to myofibril, with ATP splitting and active contraction, and that termination of the active state leads to passage of Ca^{++} from myofibril to sarcoplasmic reticulum, with cessation of ATP splitting and return of filaments to their resting state. Yet, actomyosin ATPase is not significantly affected either by the addition of Ca^{++} or its removal with the chelating agent EGTA; and this paradox has had tremendous import.

It was observed by Perry & Grey (249) that "natural actomyosin," extracted as a crude complex from muscle, displays Mg^{++}-ATPase activity that is diminished by EGTA, whereas "synthetic actomyosin," prepared from purified actin and myosin, displays Mg^{++}-ATPase activity that is insensitive to EGTA. That this effect is the result of the removal of Ca^{++} rather than EGTA itself was clearly shown by Weber & Winicur (250), among others (see 8, 9). The inhibitory effect of EGTA on Mg^{++}-ATPase and superprecipitation of natural actomyosin provides in a sense an in vitro equivalent to muscle relaxation in vivo; and in a crucial experiment, Ebashi (251) demonstrated that the EGTA-sensitizing factor comprises a tropomyosinlike protein, as shown by the restoration of EGTA sensitivity to synthetic actomyosin (251) or trypsin-degraded preparations of natural actomyosin (233). Comparable findings were obtained after desensitization of natural actomyosin by repeated washings at low ionic strength (252).

Ebashi & Kodama (235, 253) then identified the EGTA-sensitizing protein as a complex of tropomyosin and troponin. That is, while troponin and tropomyosin alone do not confer EGTA sensitivity on actomyosin, troponin and tropomyosin together inhibit superprecipitation of actomyosin in the presence of EGTA, and this inhibition is removed by Ca^{++} (240, 253). Other studies (254) demonstrate that tropomyosin alone inhibits Ca^{++}-ATPase of actomyosin, but does not affect Mg^{++}-ATPase of actomyosin. The key to interpretation of these and other fairly complicated interactions of troponin, tropomyosin, and actomyosin was provided by the finding of Ebashi, Ebashi & Kodama (238) that Ca^{++} binds strongly to troponin alone. This would suggest that Ca^{++} may induce a conformational change in troponin that is mediated through tropomyosin to an F-actin site which in turn initiates cyclic interaction with myosin (238). In any event, binding and detaching of Ca^{++} to and from troponin would be of primary importance in regulating muscular contraction (9, 238). This general picture has been substantiated in an elegant way by Tonomura et al (311) in studies involving the electron paramagnetic resonance spectra of troponin, spin-labeled tropomyosin and spin-labeled actin, in which fluctuations of Ca^{++} in micromolar concentration are transmitted to tropomyosin only in the presence of troponin, and to actin only in the presence jointly of troponin and tropomyosin.

The story becomes even more elaborate, for Hartshorne & Mueller (242) shortly demonstrated that each of the two major fractions of troponin has unique roles in the contractile process. Troponin B inhibits Mg^{++}-ATPase of actomyosin, but this effect (unlike that of intact troponin) is insensitive to Ca^{++}. Addition of tropomyosin greatly enhances the inhibitory effect of troponin B, without restoring Ca^{++} sensitivity. In contrast, troponin A shows no significant effect on the Mg^{++}-ATPase of actomyosin, whether or not tropomyosin or Ca^{++} is present; however, addition of troponin A to the troponin B-tropomyosin-actomyosin system diminishes the extent of ATPase inhibition in the absence of Ca^{++}, and activates ATPase in the presence of Ca^{++}. Thus, the troponin-tropomyosin complex provides a sensitive regulatory system in which troponin B inhibits actomyosin ATPase, tropomyosin enhances this inhibition, and troponin A activates ATPase, especially after interaction with Ca^{++} (241, 242).

With respect to the mechanism of troponin-tropomyosin regulation of actomyosin ATPase, Dancked (255) has surmised that troponin-tropomyosin inhibits Mg^{++}-ATPase by increasing the affinity of actomyosin for Mg^{++}-ATP and decreasing the rate of hydrolysis. However, Eisenberg & Kielley (256) recently reported that inhibition involves diminished binding of heavy meromyosin to F-actin, without effect on the rate of hydrolysis. Nor is controversy ended, for Dancker (257) has also presented evidence suggesting that Ca^{++} exchange during contraction involves myosin rather than troponin.

OTHER MYOFIBRILLAR PROTEINS

Although a number of proteins have been reported isolated from muscle extract, some of these are very likely contaminants or denaturation products of the major myofibrillar proteins. The early work has been summarized elsewhere (53), and here we shall simply mention several proteins that seem to have well-defined functional roles in the myofibril.

α-Actinin was isolated by Ebashi & Ebashi (258) from crude preparations of actin. α-Actinin accelerates the superprecipitation of actomyosin (258), although it is not absolutely essential for this reaction (259); and it forms a specific complex with F-actin (259–262), in which α-actinin competes with tropomyosin for actin-binding sites (262, 263). α-Actinin contains three different components, with sedimentation coefficients 6S, 10S, and 26S (264). The 6S component retains the functional activity of actinin (9, 263), and has been identified within the Z-band (265). It has been concluded that α-actinin (9, 261, 265) and its 6S component (265) in particular, interact with F-actin in the formation of networks within the Z-band; and electron microscopic evidence has been reported for bridging of α-actinin between thin filaments (266).

Another protein, β-actinin, was isolated by Maruyama (267) from crude preparations of actin. This protein fraction appears to inhibit cross-linking by F-actin (268) and regulate the overall length of the F-actin double helix (269, 270); however, structural characterization has not yet been reported.

On electron microscopy, there appears to be a network linking thick filaments at the M-line (231, 271–273). A distinct M-line protein has been identified by immunochemical methods (271, 272), and there is preliminary account of its isolation (272).

OVERVIEW AND CLINICAL IMPLICATIONS

Table 1 summarizes structural details of the major myofibrillar proteins. According to current concepts (9), excitation leads to transfer of Ca^{++} from sarcoplasmic reticulum to troponin sites along the thin filament, initiating conformational changes in troponin, tropomyosin, and actin, the outcome of which is transient actin-myosin binding and activation of myosin ATPase. The presence of two symmetric protomers in myosin and the preliminary evidence for negative cooperativity between them with respect to nucleotide binding (see 7), presteady-state ATPase (143–149), and kinetics of actomyosin ATPase (183) would suggest that the primary contractile event involves cyclic interactions of each myosin protomer with thin-filament sites and free ATP (7).

According to the sliding-filament theory (1–5), contraction of striated muscle involves interdigitation of thick and thin filaments without change in filament length, and contractile force is generated by cross-bridges moving independently about a rigid thick-filament core. The sliding-filament theory is widely accepted, but it is appropriate to indicate some recent objections. For example, Mandelkern & Villarico (274) have argued that contraction of glycerinated muscle fibers shows characteristics of a cooperative structural transition, as indicated by thermodynamic properties (274–276) and loss of α-helical conformation on wide-angle X-ray diffraction (277). This interpretation has been disputed vigorously (278), although the essential evidence remains unexplained according to the sliding-filament theory. More recently, based on reinterpretation of well-established low-angle X-ray diffraction, polarization, and energetic data, Dreizen & Gershman (7) have proposed a torsional model for contraction, postulating that cross-bridges interact in a helical path with consequent winding of the thick-filament core within a rigid thin-filament lattice. A torsional model would be consistent with thermodynamic data (274–276) and wide-angle X-ray diffraction patterns (277) on glycerinated muscle, and this model would also predict shortening of the thick filament during marked contraction, a phenomenon that has never been convincingly explained by the sliding-filament theory (cf. 279).

With respect to biosynthesis of the myofibrillar proteins, Heywood, Dowben & Rich (280) have reported in vitro synthesis of embryonic chick muscle myosin on large polysomes (50–60 ribosomes), that yield a single population of messenger RNA which directs de novo synthesis of myosin (281). Actin, myosin, and tropomyosin appear to be synthesized on monocistronic messenger RNA molecules, with synthesis of actin before myosin before tropomyosin (282). Electron microscopy (283, 284) shows sequential formation of randomly arranged thin filaments, thick filaments, and organized myofibrils. The question of postnatal biosynthesis is controversial, with recent reports of longitudinal splitting of myofibrils (285) and accumulation of newly synthesized protein about existing myofibrils (286).

What relevance do these basic molecular mechanisms of contractility have to clinical medicine? A priori, there might be genetic variations of the contractile proteins which lead to impaired biosynthesis or to the production of abnormal proteins. In the case of myosin, the evidence for microheterogeneity and related differences in ATPase activity clearly implies the likelihood of genetic variations. This matter has not yet been explored in depth, however, except for reports that myosin from dystrophic muscle has no grossly abnormal features (287–289).

There is some evidence that cardiac myosin undergoes disease-related changes. Several laboratories have described a decrease (~30 percent) in myofibrillar ATPase and actomyosin ATPase in cardiac muscle following hypertrophy (290, 291), failure (292–294), and aging (295); and this effect has been related to myosin itself in studies on cardiac myosin of dogs with naturally occurring heart failure (296).

Myosin or myosin biosynthesis may also undergo changes under physiological control. For example, myosin ATPase is decreased (~30 percent) in heart muscle of adrenalectomized cats (297) and heart and skeletal muscle of hypophysectomized rats (298), with return to control values after hormone replacement. Administration of thyroxine is accompanied by increase of myosin ATPase and minor changes in amino acid composition and optical rotatory dispersion of cardiac myosin in guinea pigs (299). In addition, cross-innervation experiments suggest that neural regulation may affect the specificity of myosin in red and white skeletal muscle (300).

The small differences reported in all these experiments are controversial (see 12). Nevertheless, we believe the overall evidence is sufficient to implicate some change(s) in myosin under several pathological and physiological conditions, although it remains uncertain whether the observed differences result from variations in primary structure or subunit composition of myosin, or from denaturation of myosin in situ or during purification.

There is no reported evidence of microheterogeneity of actin, tropomyosin, or troponin, nor of possible changes in their functional activity under different physiological or pathological conditions. Actin would seem an unlikely candidate for such change, as the F-actin structure (4) and amino acid content of actin (301) are fairly stable throughout evolution and actin may be traced to primitive motile forms without much evidence of specificity (302). On the other hand, the tropomyosin-troponin complex comprises an exquisitely sensitive regulatory system, and here might be unrecognized structural differences having drastic effects on muscular function.

Finally, if we may speculate, it would seem that studies of the myofibrillar contractile proteins are about to enter the promised land of clinical investigation, as reached earlier by hemoglobin and the immunoglobulins. Within the next few years, we may anticipate decisive evidence that at least some kinds of cardiac diseases are associated with or derive from abnormal myofibrillar proteins.

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INTESTINAL DIGESTION AND MALDIGESTION OF DIETARY CARBOHYDRATES[1]

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In the past ten years much new information about the digestion and absorption of carbohydrates has come forth which now makes it possible to interpret alterations in intestinal disease. The initial stimulus for this was the dramatic demonstration from Crane's laboratory that the luminal brush border could be separated from the remainder of the intestinal epithelial cell (1), and that this organelle contains the carbohydrases that hydrolyze the dietary disaccharides sucrose and lactose (2) as well as the oligosaccharide products released by action of pancreatic amylase on starch. Hence, the surface of the intestinal cell plays a critical role not only in absorption but also in digestion of dietary carbohydrate.

DIGESTION AND ABSORPTION IN NORMAL MAN

CARBOHYDRATES IN MAN'S DIET

Starch, sucrose, and lactose are the carbohydrates of dietary importance and together they account for about 50 percent of man's daily caloric intake (3). Table 1 lists the food source and typical amounts of each carbohydrate ingested daily in the western world. In other parts of the world, carbohydrates are a very inexpensive source of calories and may provide a much higher proportion of ingested food.

INTRALUMINAL DIGESTION

Despite the existence of salivary amylase and the low pH of gastric contents, little happens to ingested starch or disaccharides until they enter the duodenum. Starch is hydrolyzed by α-amylase which is secreted via the pancreatic duct at high concentrations into the mid-duodenum. As an endoenzyme, α-amylase splits the interior 1–4 glucose-glucose linkages of the starch molecule but is inactive against the outermost terminal links of this glucose polymer. Hence, the final breakdown products of the intraluminal amylase action on the type of starch (amylose) having solely 1–4 glucose linkages are a disaccharide (maltose) and a trisaccharide (maltotriose)

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TABLE 1. DIETARY CARBOHYDRATES

Type of carbohydrate	Typical source	Amount[a] in diet (grams)	% of total CHO
Starch (amylopectin, amylose)	Potatoes, rice, corn, bread	210	60
Sucrose	Table sugar, desserts	105	30
Lactose	Milk, ice cream, milk products	35	10

[a] Values based on Hollingsworth & Greaves (3).

(4, 5). In addition, branched saccharides of varying molecular size are also formed as final hydrolytic products because some starch (amylopectin) has 1–6 branching points along the 1–4 glucose-linked straight chain. α-Amylase is unable to hydrolyze these branching links and is inactive against 1–4 links adjacent to the branching point (5). These branched oligosaccharides released from starch are the α-dextrins which vary in molecular size, having an average of 8 glucose units.

Unlike starch, lactose and sucrose are not acted upon by intraluminal duodenal enzymes.

DIGESTION BY THE INTESTINAL EPITHELIAL CELL

Table 2 lists the form in which dietary carbohydrate is presented to the intestinal mucosa at the duodenal-jejunal junction and the apparent rate-

TABLE 2. INTESTINAL DIGESTION OF CARBOHYDRATE

Dietary source	Form presented to brush border	Enzyme[a]	Monosaccharide products	Apparent rate-limiting step
Starch	Maltose, maltotriose, α-dextrins	Maltase α-dextrinase (isomaltase)	Glucose	Transport
Sucrose	Sucrose	Sucrase	Glucose, fructose	Transport
Lactose	Lactose	Lactase	Glucose, galactose	Hydrolysis

[a] Whereas there appear to be several enzymes responsible for maltase activity, recent evidence suggests that brush border contains only a single α-dextrinase, one sucrase, and one lactase. The exact location of three other maltases in the intestinal cell is uncertain as is their possible digestive role.

limiting step in the overall process of cellular digestion and absorption. The enzymes that hydrolyze these oligosaccharides are located within the membrane of the brush border surface of the columnar epithelial cells of the small intestine (2) and are most active in the jejunum. Hydrolysis occurs rapidly enough in most instances so that sufficient monosaccharide is released to saturate the hexose transport mechanisms (6). However, lactase activity is lower than that of other disaccharidases in normal man so that the maximal hydrolytic rate of lactose is too slow to saturate the glucose-galactose transport mechanism (6). In other words, the hydrolytic step appears to be rate-limiting for the overall process of hydrolysis and absorption of lactose. On the other hand, the other oligo- and disaccharides are split so rapidly by their brush border enzymes that monosaccharide transport becomes the apparent rate-limiting step. These facts are undoubtedly important for the carbohydrate maldigestive states that are discussed below.

MECHANISMS OF INTESTINAL TRANSPORT

Uptake by the intestinal cell is reserved specifically for the monosaccharides released at the brush border surface, there being no known mechanism for quantitative entry of disaccharides into the epithelial cell. Although trace amounts (50–300 mg per day) of disaccharide may diffuse past the mucosal barrier after a carbohydrate-containing meal (7), there is no hydrolysis in other body organs and the sugar is lost in the urine. This minimal loss is not of any importance to the body economy.

Active transport.—Glucose and galactose, being of similar structure, are both transported by a single process that requires energy and Na^+ and demonstrates saturation kinetics, suggesting that binding and release phenomena may occur at the cell surface and allow these simple sugars to enter the otherwise impermeable intestinal cell (8, 9). The process allows these sugars to accumulate intracellularly at concentrations higher than those in the lumen.

Facilitated diffusion.—Fructose, one of the products released upon hydrolysis of sucrose, is the only other monosaccharide of dietary importance, and it appears to have a transport mechanism separate from that for glucose that does not require Na^+ (10) but allows the sugar to enter the cell and move down its own concentration gradient.

DEFECTIVE DIGESTION AND ABSORPTION

MALDIGESTION OF STARCH. AN ENTITY?

The pancreas secrets α-amylase in amounts at least ten times that required to completely digest starch intraduodenally to its oligosaccharide constituents. It is often assumed that patients with pancreatic exocrine insufficiency have a markedly depressed output of all digestive enzymes, producing severe maldigestion for all foods. However, even in patients with severe fat maldigestion due to the lipase deficiency in chronic pancreatitis, there is still sufficient amylase to hydrolyze all of the ingested starch (11). This discrepancy between fat and carbohydrate digestive capacity in severe inflammatory disease of the pancreas defies a ready explanation. Except in the first month or so of life when amylase is not yet produced, or in complete obstruction of the pancreatic duct, it is unlikely that starch maldigestion is of clinical significance. Nevertheless the preservation of pancreatic amylase at physiologically active levels even in extensive pancreatic disease suggests that starch may constitute an excellent source of usable calories in pancreatic insufficiency.

OLIGO- AND DISACCHARIDASE DEFICIENCIES OF THE SMALL INTESTINE

The clinical syndrome.—Although for many years pediatricians have noted intolerance to milk manifested by diarrhea, vomiting, and failure to thrive in the newborn period, the syndrome was commonly believed to be caused by a reaction, perhaps allergic, to milk protein. It was not until a few years ago, with the demonstration of disaccharide intolerance and concomitant acid diarrhea in these children (12–14), that the medical world was alerted to the role of disaccharide maldigestion in the pathogenesis of milk intolerance. Shortly thereafter, adults were discovered with symptoms of abdominal bloating, gaseousness, and watery diarrhea after ingestion of milk, and were found to have reduced concentrations of lactase in their intestinal mucosal biopsies (15, 16). Typically patients note abdominal noises, vague discomfort or nausea, mild distention, cramping pain, and diarrhea after ingestion of 15 to 100 g of the offending sugar. Young children occasionally vomit. When the food containing the specific saccharide is avoided, symptoms do not occur. Stools are usually watery and, in children, often have a sour odor.

As outlined in Figure 1, diarrhea is produced in disaccharidase deficiency because the disaccharide, having no brush border surface enzyme to hydrolyze it and possessing no mechanism of entry into the intestinal cell, remains in the intestinal lumen and attracts water by virtue of its osmotic effect (17). The greater intestinal fluid load then appears to stimulate peristalsis, thereby hastening the transit time (18). An accompanying mild malabsorption of other foods (19) and drugs may also occur (20). In addition, some of the disaccharide is metabolized to lactic acid in the lower ileum and colon, further increasing the osmotic force and perhaps hampering the colonic absorption of water and electrolytes (21).

THE DIAGNOSIS OF INTESTINAL DISACCHARIDASE DEFICIENCY

When the typical syndrome of abdominal distention and diarrhea follows 30 to 60 minutes after the ingestion of the offending sugar, deficiency of the particular sugar-splitting enzyme should be suspected. The most reliable way of establishing the diagnosis is the demonstration of reduced levels of

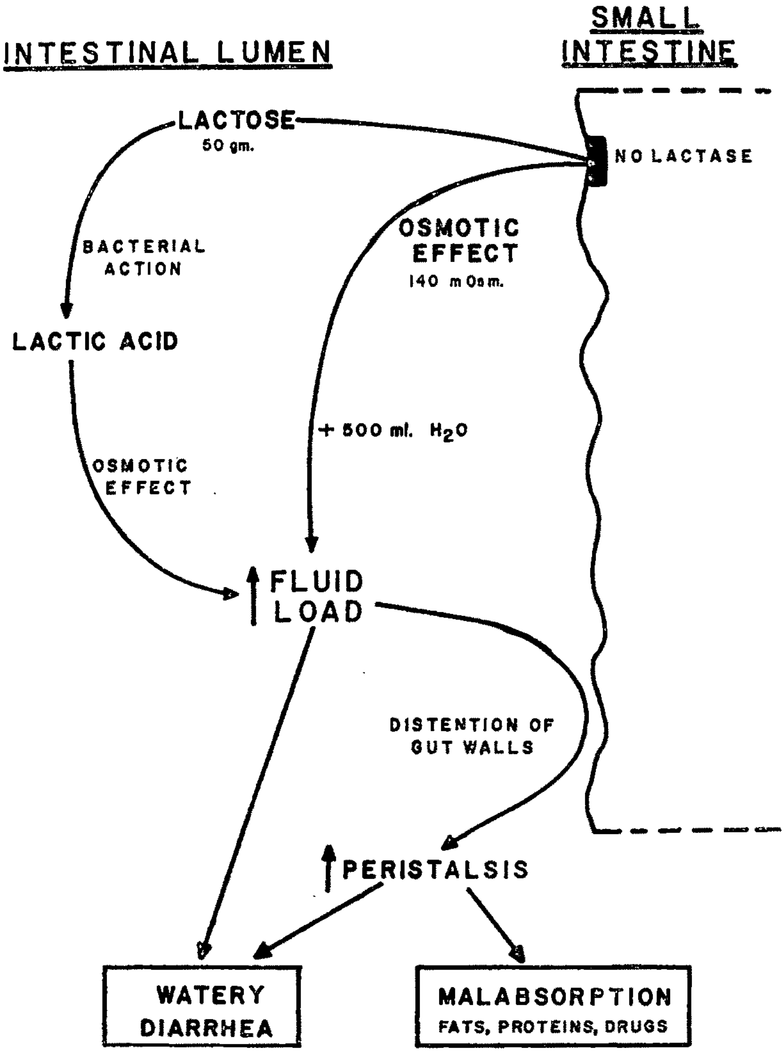

FIGURE 1. Schematic outline of the pathogenesis of lactose intolerance. See text for discussion.

the disaccharidase in assay of the small intestinal biopsy (15, 16). Only a small specimen (3 mg) of mucosa from a peroral biopsy is required, and the tissue can be preserved for many weeks in an ordinary freezer (−20°C) prior to analysis (22). Typical normal values are given in Table 3. Values below these are usually associated with disaccharide intolerance.

Unfortunately, it may be hazardous to perform an intestinal biopsy on small children (23). However, a small intestinal X ray taken after ingestion

TABLE 3. NORMAL VALUES OF INTESTINAL DISACCHARIDASES[a]

	Lactase	Sucrase	Isomaltase	Maltase	Sucrase/ lactase
U/gram protein	45 (15–95)	93 (40–165)	99 (50–160)	262 (128–461)	2.1 (1.0–4.5)
U/gram wet weight	3.5 (1.0–6.5)	8.1 (3.5–14)	8.3 (3.8–14)	20 (10–36)	2.3 (1.0–4.5)

[a] From combined data on 50 patients from Puerto Rico and the United States. U = μmoles/min mean values with range in parentheses.

of a disaccharide-barium meal may readily identify disaccharidase deficiency because of the dilution of barium and dilation of intestinal loops (24, 25), and may constitute the best approach to establishing the diagnosis when a biopsy is contraindicated. An important supportive finding in the diagnosis is the abnormal clinical response to an oral load of the offending disaccharide marked by gaseousness, bloating, and diarrhea within 30 minutes to 4 hours.

The disaccharide oral tolerance test has been widely used (26) as a criterion for diagnosis but is less satisfactory than biopsy assay, particularly when lactose is given, because of the smaller rise in blood glucose concentration than is found with other disaccharides, reflecting the relatively low digestive capacity for this sugar in normal man (6). Hence, false positive ("flat") tolerance tests may occur in as high as 30 percent of normal subtests (27). Nevertheless, the finding of a blood glucose rise of less than 20 mg/100 ml (preferably only 10 mg/100 ml) 15 to 60 minutes after ingestion of 50 g (2.0 gm/kg for children) of the disaccharide coupled with the production of diarrhea, constitutes presumptive evidence of enzyme deficiency (28).

There is another pitfall in the use of oral tolerance tests since glucose-galactose malabsorption, a rare entity, is associated with flat oral tolerance curves for all dietary disaccharides because all contain glucose. Should this be a consideration, administration of 25 g glucose + 25 g galactose orally will produce a flat curve in monasaccharide malabsorption but will show a normal increase in blood sugar in disaccharidase deficiency.

OLIGO- AND DISACCHARIDASE DEFICIENCIES: ENZYME DEPRESSION IN DISEASE STATES

Since these enzymes are constituents of the microvillar surface of the intestinal cells, they can be expected to be affected by diseases that damage the small intestinal epithelium. Table 4 outlines diseases of the small intestine in which brush border disaccharidases have been shown to be depressed. In most instances, there is gross histological alteration of the jeju-

TABLE 4. DISACCHARIDASE DEFICIENCY IN DISEASE STATES[a]

Diagnosis	No. of Patients	% lactase-deficient	% sucrase- or maltase-deficient	References
		Gastrointestinal disorders		
Celiac sprue				
untreated	51	100	76	(29–34)
treated	14	64	50	(34)
Tropical sprue				
untreated	46	100	72	(32, 35)
treated	48	63	0	(35)
Kwashiorkor				
treated	17	100	0	(36)
"Irritable" colon	71	38	0	(37, 38)
Infant diarrhea	10	40	30	(39)
Postgastrectomy	42	36	0	(40, 41)
		Other primary disorders		
Osteoporosis	19	47	0	(42)
Cystic fibrosis	28	25[b]	0	(43)

[a] Most patients were white with a presumed prevalence of lactase deficiency in the healthy control group of 5 to 20 percent.

[b] No lactase deficiency was found in a control group of 90 patients.

nal epithelium, including shortening of the microvilli and reduction in their numbers. Although little information is available correlating the sequence of enzyme depression and recovery in disease states, alteration in these brush border carbohydrases usually reflects the severity of the primary intestinal disease. Even so, lactase is frequently more severely reduced than other disaccharidases (Fig. 2), and it is the last enzyme to return to normal after clinical recovery of the primary illness. Indeed, in tropical sprue (35), celiac sprue (34), and kwashiorkor (36), illnesses that undergo rapid improvement with appropriate therapy, lactase remains low for a year or more in the majority of patients after they undergo a clinical remission of the primary disease, and may be permanently depressed as the sole subclinical evidence of these diseases.

There is no ready explanation for the apparent special vulnerability of lactase to intestinal injury but it may pose a knotty problem for the clinician. Since lactase deficiency may in certain instances outlast all other abnormalities when the primary disease approaches remission, it may be impossible to establish whether the enzyme deficiency is an isolated primary

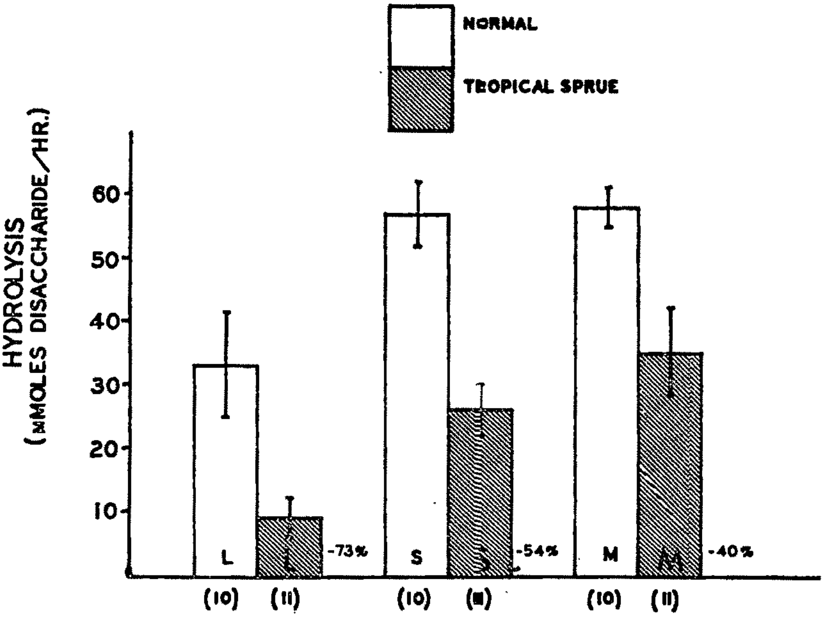

FIGURE 2. In vivo jejunal perfusion in man of a 30-cm segment of jejunum with disaccharides (70 mM) demonstrating more severe reduction in capacity to hydrolyze lactose (L) than other disaccharides (M = maltose, S = sucrose) in patients with active intestinal disease. Percentage values indicate degree of depression from normal. [Reproduced from G. M. Gray and N. A. Santiago (6) with permission of *Gastroenterology,* Williams & Wilkins Co., Baltimore, 1966.]

defect or merely the sole permanent residuum of previous or subclinical intestinal disease. Certainly, it is mandatory to rule out the possibility of a serious underlying intestinal disease in patients with an isolated lactase deficit.

Several diseases that do not directly involve the intestine may be associated with an increased frequency of lactase deficiency, but it is very difficult to substantiate more than chance occurrence when there is such a high prevalence of deficiency of the enzyme in healthy populations (see below and Table 5). Nevertheless, there does appear to be a true increase in incidence of lactase deficit in osteoporosis (42) and cystic fibrosis (43) (Table 4). Nearly half of patients with osteoporosis are lactase-deficient and have ingested low amounts of milk and calcium for many years, suggesting that the bone disease may be a result of long-term avoidance of milk products. Whereas patients with cystic fibrosis and normal lactase activity are very young (average age 3 years), those with lactase deficiency associated with this disease are older (average age 11 years) (43), suggesting that the defect may be acquired, perhaps as a result of the chronic infection and inani-

TABLE 5. PREVALENCE OF LACTASE DEFICIENCY IN HEALTHY POPULATIONS

Group	Number tested	Location	% lactase-deficient[a]	Reference
		By intestinal biopsy assay		
White	20	Baltimore	5	45
	14	San Juan, P. R.	14	6
	100	Rochester, Minn.	10	46
	93	Chicago	19	47
	700	Copenhagen	3	48
	50	Washington, D. C.	20	32
	145	Oklahoma	19[b]	49
American Negro	20	Baltimore	70	45
	24	Chicago	75	47
African Bantu	40	Uganda	50	50
Puerto Rican	28	Puerto Rico	21	6
Oriental	11	Rochester, Minn.	100	51
		By oral lactose tolerance		
White	23	Australia	0	52
	17	Greek Cypriots	88	53
Aborigine	44	Australia	85	54
Chami Indian	24	Columbia, S. A.	100	55
Chinese	15	Australia	87	56
	10	Baltimore	95	57
Eskimo	32	Greenland	88	58
Indian	22	Northern India	55	59
Filipino	10	Baltimore	95	57
Thai	140	Thailand	97	60

[a] Lactase activity below 1.0 U/g tissue or 15 U/g protein is generally accepted to be indicative of lactase deficiency. Hence, percentage values given may differ in some instances from those in the reference.

[b] Hospitalized patients without small intestinal disease.

tion commonly seen in cystic fibrosis.

LACTASE DEFICIENCY IN INFANTS AND CHILDREN

Man develops full capacity to digest lactase in utero (44) and most newborns have no difficulty digesting the lactose in milk. Nevertheless, a rare child does demonstrate severe acid diarrhea ($pH < 6.0$) which contains undigested lactose. Vomiting may also accompany the diarrhea. Whereas it is not often possible to perform intestinal biopsies on such small children, an oral load (2 mg/kg body weight) of lactose will reproduce the clinical syndrome and is associated with failure of the blood sugar concentration to rise. This type of lactase deficiency is often referred to as "congenital," but there are few long-term studies and many children develop a normal lactase within months to a year or so after the deficiency is first uncovered (39), suggesting that temporary intestinal injury or delayed development of lactase may occur in such infants.

IDIOPATHIC LACTASE DEFICIENCY IN ADOLESCENTS AND ADULTS: GENETIC OR ENVIRONMENTAL ETIOLOGY?

By far the commonest type of disaccharide deficiency is lactase deficit developing in adolescent or young adults. There is usually no history of intolerance in early childhood and those affected later learn to avoid symptoms by eliminating milk. When seen by a physician many years later, they often have only a vague recollection of the reasons for discontinuing milk drinking even though they can be shown to be intolerant to as little as one glass of milk (13 g of lactose).

The lactase deficiency state is present in a remarkably high proportion of many racial groups (Table 5) so that half the world's population may be lactase-deficient as young adults. Even in healthy people of northern European heritage the prevalence of lactase deficiency is remarkably high (up to 20 percent). This has prompted many authors to state that lactase deficiency is the normal condition in adults and that some racial groups acquired the adult form of this enzyme as a result of a genetic mutation (57). However, this hypothesis would require that possession of intestinal lactase provide a selective advantage for a population group, and there is no evidence that the European white population ever depended upon milk as an obligate nutritional source. At any rate, careful multigeneration genetic studies must be carried out before lactase deficiency can be said to be a heritable disorder.

Induction of lactase by dietary lactose has been proposed as a mechanism of maintaining activity of the enzyme (61), but correlation of enzyme activity with milk drinking may only reflect a pre-existing tolerance or intolerance to lactose and attempts to induce the enzyme by milk drinking have been unsuccessful in man (60). Furthermore, patients with galactosemia, who chronically avoid milk because of the galactose content, maintain their lactase at normal levels (62).

The vast majority of nonwhite racial groups show a high prevalence of lactase deficiency in adult members (Table 5), but it is notable that they differ from white people of Northern European origin in two important respects besides race. They have markedly different diets, frequently with moderate-to-severe malnutrition, and there is a higher prevalence of intestinal disease, especially in infants and children (63). Considering the persistence of lactase deficiency in subclinical intestinal disease, it is entirely possible that racial differences are related mainly to dietary and environmental variations rather than to genetic alterations. Persistent lactase deficiency in adults may reflect continuous exposure of the intestine to subclinical injury or serious intestinal insult in the remote past.

TREATMENT OF DISACCHARIDASE DEFICIENCIES

If there is an underlying primary disease, appropriate therapy often results in return of the enzymes to normal. If the deficiency is permanent, whatever the cause, the simplest and most effective therapy is elimination of the offending carbohydrate from the diet. Supplementation of the diet with the missing enzyme also appears to be effective (64), but it is cumbersome and expensive and probably interferes more with the patient's life than simple dietary exclusion.

COMBINED SUCRASE AND α-DEXTRINASE (ISOMALTASE) DEFICIENCY

There are about 75 documented cases of this malady from many different areas of the world even though it has been recognized for only a few years (65–68). It appears to be inherited as an autosomal recessive and all patients with sucrase deficiency have also been shown to have markedly depressed isomaltase activity. The biochemical explanation for the combined defect rests on the fact that these two activities appear to be located at independent sites on the same protein molecule since it has not been possible to separate them physically. Nevertheless, the two enzyme sites do seem to function completely independently on their respective substrates. The gene involved probably is responsible for synthesis of the total protein molecule or perhaps is necessary for assembly of the two enzyme units into a single package.

Symptoms essentially identical to those found with lactase deficiency are first noted in early childhood in response to the intake of table sugar and candies. The cause is readily recognized by parents and elimination of dietary sucrose produces complete relief of symptoms. The family physician may obtain the history incidentally many years later since sucrose is not often an obligate source of calories. Despite the fact that α-dextrinase deficiency is also present, intolerance to starch occurs uncommonly, probably because the unhydrolyzed α-dextrins, being of large molecular weight (average 1500), have only 10 to 20 percent of the osmotic force of sucrose per unit weight.

Curiously, patients with sucrase-dextrinase deficiency usually are later able to eat several grams of sucrose as young adults without developing symptoms even though they do not appear to regain enzyme activity. Perhaps this increase in capacity is related to growth of the small intestine so that, while enzyme concentration remains low, the total enzyme activity of the gut increases sufficiently to allow digestion of some sucrose.

Therapy consists of elimination of sucrose and is uniformly successful except in areas of the world where sucrose is a major source of calories.

GLUCOSE-GALACTOSE MALABSORPTION

This specific defect in monosaccharide absorption must be considered together with the carbohydrase deficiencies because the clinical syndrome is identical with that in disaccharidase deficiency.

More than 20 cases have now been reported (69–73) and an autosomal recessive genetic lesion is supported by a Swedish family study over 10 generations in which there was considerable intermarriage of first and second cousins (74).

Children demonstrating intolerance to all common dietary carbohydrates should be suspected of having the lesion. Flat oral tolerance curves are found with all carbohydrates except fructose, which utilizes a different entry and transport mechanism than glucose. Failure of blood sugar to rise after ingestion of glucose + galactose distinguishes this entity from disaccharidase deficiency. Furthermore, incubation of an intestinal biopsy with glucose shows absence of the normal capacity to accumulate the sugar at concentrations higher than in the medium The finding of glucosuria (250–1000 mg/ml) should also alert the physician to the diagnosis since all patients appear to have an associated mild defect in the renal tubular reabsorption of glucose. The usual dietary carbohydrates must be avoided since they all contain glucose. This often produces a serious problem in nutrition which can only be surmounted by using fructose as a principal source of carbohydrate. Some patients are able to tolerate moderate amounts of sucrose, probably because one of its monosaccharide constituents is fructose.

The exact abnormality in glucose absorption is unknown but there is indirect evidence suggesting that the site of the defect can be localized. Na^+ transport is believed to be necessary for active glucose transport and the ion itself may provide the driving force for glucose accumulation by virtue of its active extrusion out of the lateral and basilar margins of the intestinal cell. Because transport of this ion is normal in glucose-galactose malabsorption, it can be inferred that the defect in monosaccharide malabsorption is localized to the entry step rather than to the mechanism of accumulation.

SUMMARY

Dietary carbohydrates constituting a most important source of nutrition, are digested and absorbed by a series of complex processes beginning with starch hydrolysis within the small intestinal lumen by pancreatic amylase. Further digestion of released oligosaccharides as well as hydrolysis of sucrose and lactose occurs on the intestinal cell surface under the influence of constitutive brush border enzymes. The intestinal cell then transports the final hexose products either by an active process (for glucose and galactose) or by a passive one (fructose).

Theoretically, a defect in the handling of carbohydrate could occur anywhere in this scheme, but deficiency of amylase practically never occurs in pancreatic disease. Hence, deficient digestion or absorption of carbohydrate, whether primary or secondary, can almost always be localized to a defect in the enzymic or transport capacity of the small intestinal surface cell. Considering this, further study of the digestive and absorption function of the intestinal cell is most likely to provide essential information for a clearer understanding of alteration in disease processes.

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